Title:

Transcription regulation of rRNA gene by a multi-functional nucleolar protein, B23/nucleophosmin through its histone chaperone activity

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ABSTRACT

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 the rRNA gene transcription is likely to be dependent on the dynamic conversion of the chromatin structure.   Previously, we identified B23/nucleophosmin, a multi-functional 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 is also shown that B23 has the histone chaperone activity.   Here, we examined the effect of B23 on the rRNA gene transcription.   B23 was found to be associated with the rRNA gene chromatin.   siRNA-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 the rRNA gene transcription and the rate of cell proliferation.   These suggest that B23 is involved in the transcription regulation of the rRNA gene as a nucleolar histone chaperone.

INTRODUCTION

To ensure the protein synthesis level, growing cells require continuous ribosome supply by synthesis of ribosomal proteins and ribosomal RNA (rRNA). The transcription rate 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 nucleolar organizer region (NOR). The tandem rRNA gene repeats exist in two distinct types of chromatin, an 'open' one permissive for transcription and a 'closed' one under 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 that the epigenetic control mechanism is important for the transcription regulation of the rRNA gene. For instance, it is 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 is 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 targets these enzymatic activities to the rRNA gene promoter to form heterochromatin (18). In addition to histone modification enzymes and ATP-dependent chromatin remodeling factors, it is shown that nucleolin, one of histone chaperones 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 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, 44). 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, the access to the viral genome of the factors required for the replication and transcription is restricted. Using a *cell-free* DNA replication system using Ad core as a template, we have identified three host factors termed Template Activating Factor (TAF)-I, TAF-II, and TAF-III. TAF-I and TAF-II are found to be 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 (44). Recombinant B23 induces the structural alteration of Ad core, thereby stimulating the Ad core DNA replication (44, 49). TAF-I and NAP-1 bind to core histones directly and transfers them to naked DNA (25, 35). TAF-I is not only involved in the replication and transcription of Ad core (20, 21, 37), but also 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 chromatin template *in vitro* (14), and stimulates the transcription of a sub-set 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 (45) as do TAF-I (35) and NAP-1 (25), and the acidic regions of B23 are important for its activity (45). The amino-terminal domain of B23 shows significant similarity to nucleoplasmin, which is the first identified histone chaperone (32). Recently, it is indicated that B23 is acetylated, and acetylated B23 has the enhanced histone chaperone activity and stimulates *in vitro* transcription from chromatin templates by RNA polymerase 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 each pentamer further assembles into a decamer 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 is reasonably assumed that B23 is involved in regulation of the chromatin structure around the rRNA gene through its histone chaperone activity.

B23 is also termed as nucleophosmin, numatrin, or NPM1, and was initially identified as an abundant nucleolar phosphoprotein (47). These properties are similar to those of nucleolin, alternatively termed C23 (47). The expression level of B23 is up-regulated in association with growth factor-induced mitogenesis (12). A positive correlation has been reported among the quantity of B23 and nucleolin, the rate of Pol I transcription, and the cell proliferation rate (7). There exist two subtypes of B23 termed B23.1 and B23.2, the latter of which lacks the carboxyl-terminal region present in B23.1 (4). B23 is a multi-functional protein: It is suggested that B23 is involved in rRNA maturation through its nucleic acid binding and ribonuclease activities (9, 22). B23 is shown to play a critical role in centrosome duplication (43). 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 the rRNA

gene transcription.   We showed that down-regulation of B23 by siRNA reduces the

transcription rate of the rRNA gene.   Nucleolar chromatin immunoprecipitation 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.   Over-expression of B23ΔC reduced the transcription level

from the rRNA gene.   These results altogether suggest that B23 regulates the transcription

from the rRNA gene chromatin as a nucleolar histone chaperone.

1 **MATERIALS AND METHODS**

2

3 **Cell culture and transfection**

4   293T and $p53^{-/-}$ MEF cells were cultured at 37ºC in DMEM supplemented with 10%

5 fetal bovine serum (FBS).   HeLa cells were cultured at 37ºC in MEM supplemented with

6 10% FBS.   Transient transfection was performed using *Trans*IT-293 (Mirus) for 293T cells,

7 *Trans*IT-LT1 (Mirus) for $p53^{-/-}$ MEF, and GeneJuice (Novagen) for HeLa cells.   HeLa cells

8 were transfected with the siRNA using Lipofectamine 2000 (Invitrogen).

9

10 **Preparation of plasmids**

11   pCHA-B23.1 was prepared as previously described (44). To generate pCHA-B23ΔC,

12 a fragment of B23ΔC3 cDNA was prepared by digestion of pET14b-hB23ΔC3 (44) with *Nde*

13 I and *Bam*H I.   The fragment was then cloned in-frame to *Bst*E II- and *Bgl* II-digested pCHA

14 (44).   To generate pUC119-HurDNA, the human rRNA gene (-192~+703 relative to the

15 transcription start site (+1)) was amplified by PCR using genomic DNA purified from HeLa

16 cells   as   template.   To   amplify   the   human   rRNA   gene,   two   oligonucleotides,

17 5'-CGCGGATCCTGTCCTTGGGTTGACCAGAG-3'   and

18 5'-CCGGAATTCGCAAGTCGACAACCACTGGA-3',   were   used   as   primers.   The

19 amplified human rRNA gene fragment was cloned into the *Bam*H I- and *Eco*R I-digested

20 pUC119.   For expression of Flag-tagged B23.1, B23.1 cDNA attached by the Flag-tag was

21 excised from pBS-Flag-B23.1 (46) by *Bam*H I, and subcloned into *Bam*H I-digested pcDNA3

22 vector (CLONTECH).   To   create   pCAGGS-Flag-B23.1,   a   DNA   fragment   encoding

23 Flag-tagged B23.1 was excised from pBS-Flag-B23.1 by *Bam*H I, and subcloned into *Bgl*

1   II-digested pCAGGS vector.   To generate pET-14b-B23ΔA1, a DNA fragment of B23ΔA1

2   was   amplified   by   two-step   PCR   using   four   oligonucleotides,

3   5'-GCTAGTTATTGCTCAGCGG-3' and 5'-AGTAGCTGTGGTGAAACTCTTAAGTATA-3',

4   and   5'-TAATACGACTCACTATAGG-3'   and

5   5'-AGAGTTTCACCACAGCTACTAAGTGCTG-3', and pET-14b-B23.1 as a template, and

6   cloned into the *Nde* I and *Bam*H I-digested pET-14b.   To generate pET-14b-B23ΔA, a DNA

7   fragment of B23ΔA was amplified by two-step PCR using four oligonucleotides,

8   5'-GCTAGTTATTGCTCAGCGG-3'   and

9   5'-ACTTGCTGCTAAAGCGCCAGTGAAGAAA-3',   and

10   5'-TAATACGACTCACTATAGG-3' and 5'-CTGGCGCTTTAGCAGCAAGTTTTACTTT-3',

11   and pET-14b-B23ΔA1 as a template, and cloned into the *Nde* I and *Bam*H I-digested pET-14b.

12   To generate pBS-Flag-B23ΔA, a DNA fragment of B23ΔA was prepared by digestion of

13   pET-14b-B23ΔA with *Nde* I and *Hin*d III and cloned into the *Nde* I and *Hin*d III-digested

14   pBS-Flag.   To generate pCAGGS-Flag- B23ΔA, a DNA fragment of Flag-B23ΔA was

15   prepared by digestion of pBS-Flag-B23ΔA with *Bam*H I and cloned into *Bgl* II-digested

16   pCAGGS.

17

18   **Antibodies**

19   Antibodies used in this study were as follows: Mouse monoclonal antibody for B23

20   (Zymed) recognizes both endogenous B23.1 and B23ΔA proteins, but not B23ΔC protein.

21   For immunofluorescence assays of B23, goat polyclonal anti-B23 antibody (Santa Cruz

22   Biotechnology) was used; for histone H3, rabbit polyclonal antibody (Abcam); for acetylated

23   histone H3, rabbit polyclonal antibody (Upstate); for TBP, rabbit polyclonal antibody (Santa

Cruz Biotechnology); for 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 HA-tag, rabbit polyclonal antibody

(MBL) or rat monoclonal antibody (3F10, Roche); for Flag-tag, mouse monoclonal antibody

(M2, Sigma); for His-tag, mouse monoclonal antibody (Sigma); 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, BSA (1 mg/ml at the final concentration), and

protein A-Sepharose beads (GE Healthcare) were added to the lysate, and incubated at 4ºC for

2-4 h. The beads were washed extensively with buffer A containing 300 mM NaCl, and

proteins bound to the beads were eluted with an SDS sample buffer.   Proteins were separated

through 10% SDS-PAGE and detected by immunoblotting using anti-HA, anti-B23, anti-Flag,

or anti-histone H3 antibodies.

Pulse labeling of B23

293T cells (1 x 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 37ºC for 1 h. Cell lysates were

prepared and B23 was immuno-purified using anti-B23 antibody. Labeled proteins were

separated by a 10% SDS-PAGE and visualized by autoradiography.

Short interference RNA (siRNA) for B23

B23 Stealth RNAi select containing siB23-A (NPM1-HSS143152), siB23-B (NPM1-HSS143153), and siB23-C (NPM1-HSS143154), and control Stealth RNAi Negative control (Cat. 12935-200, Invitrogen) were introduced into HeLa cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 24 h post transfection, the medium was replaced, and cells were harvested at 72 h post siRNA introduction. Proteins in HeLa cell lysates was separated by a 10% SDS-PAGE and detected by immunoblotting.

Total RNA was prepared by RNeasy mini kit (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'-GCCTTCTCTAGCGATCTGAGAG-3' and 5'-CCATAACGGAGGCAGAGACA-3' corresponding to the 5' external transcribed spacer of rRNA as described (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 a primer set, ATGGGTCAGAAGGATTCCTATGT-3' and 5'-GGTCATCTTCTCGCGGTT-3'.

For immunofluorescence, HeLa cells transfected with siRNA on glass slides were permeablized 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 chromatin immunoprecipitation

Preparation of nucleoli and nucleolar ChIP were carried out according to the protocol described previously (42). Nucleoli were prepared from 6 x 10^6 cells pre-fixed by formaldehyde (0.25% for 10 min at 37ºC). Nucleolar ChIP assays were carried out according to the manual of the ChIP assay kit (Upstate). Quantitative PCR was carried out using FullVelocity SYBR Green QPCR Master Mix (STRATAGENE). PCR reactions were carried out using primer sets corresponding to the rRNA gene indicated in Table 1.

Run-on assay

Transcriptionally active nuclei were prepared from 293T cells (1 X 10^6) by treatment with NP-40 buffer (10 mM Tris-HCl [pH7.9], 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% NP-40) and resuspended in 30 µl of glycerol stock buffer (10 mM Tris-HCl [pH7.9], 0.1 mM EDTA, 3 mM $MgCl_2$, and 40% glycerol), and stored at –80ºC until use. The isolated nuclei (30 µl) were mixed with 30 µl of a reaction buffer containing 10 mM Tris-HCl [pH7.9], 300 mM KCl, 5 mM $MgCl_2$, 0.5 mM each A, C, UTP, 50 µCi [α-^{32}P]GTP (~400 Ci/mmol) (Amersham), and 40 units RNase inhibitor (TOYOBO), and incubated at 30ºC for 30 min. Genomic DNA was digested with 200 units of DNase I at 30ºC for 10 min followed by incubation with 100 µg proteinase K at 42ºC 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 using the Image Gauge Program (Fuji-film).

***In vitro* histone binding assay**

11

1   His-tagged B23.1, B23ΔA, and B23ΔC proteins were prepared as described (44).

2   His-B23.1 and His-B23ΔC proteins were mixed at appropriate ratios and precipitated by cold

3   acetone.   The precipitated proteins were denatured in a guanidine buffer (50 mM

4   HEPES-NaOH [pH7.9], 150 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.1% NP-40, and 6 M

5   Guanidine-HCl) at room temperature for 15 min and renatured by dialysis against buffer

6   containing 20 mM HEPES-NaOH [pH7.9], 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1

7   mM PMSF, and 10% Glycerol.   Renatured B23 proteins were mixed and incubated at 4ºC

8   for 30 min with core histones purified from HeLa cells in 50 μl of 50 mM Tris-HCl [pH7.9],

9   270 mM NaCl, 1 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mg/ml BSA, and 0.1% NP-40.

10   Immunoprecipitation was carried out using anti-His or anti-B23 antibodies.   Precipitated

11   proteins were analyzed by a 12.5% SDS-PAGE and visualized by silver staining and

12   immunoblotting using anti-His and anti-histone H3 antibodies.

13

14   **Nucleosome assembly assay**

15   Supercoiled plasmid DNA (pCAGGS) was relaxed by incubation with

16   topoisomerase I (TaKaRa) at 37ºC for 30 min.   The supercoiling assay was performed

17   essentially as described (24).

12

1   **RESULTS**

2

3   **Relationship between the cell growth rate and the rRNA gene transcription level**

4   The relationship among the cell growth rate, the transcription rate of the rRNA gene,

5   and the expression level of B23 was examined using 293T cells maintained in the presence of

6   either 1% or 10% of serum.   The expression level of B23 was examined by pulse-labeling

7   with [^{35}S]methionine.   The pulse-labeled B23 was immunoprecipitated with an anti-B23

8   antibody and analyzed through a 10% SDS-PAGE followed by autoradiography.   The

9   expression level of B23 was approximately four fold higher in the presence of 10% serum

10   than in the presence of 1% serum at 48 h (Fig. 1A, lanes 3 and 4).   This result is consistent

11   with the report that the expression of B23 is associated with growth factor-induced

12   mitogenesis in Swiss 3T3 cells (12).   The expression level of nucleolus-related proteins was

13   examined in cells maintained in the presence of 1% or 10% serum by immunoblotting using

14   anti-UBF, anti-nucleolin, anti-fibrillarin, and anti-β-actin antibodies.   The protein expression

15   level of UBF and nucleolin, both of which are known to be involved in the transcription of the

16   rRNA gene, was reduced in the presence of 1% serum compared with that of 10% serum at 48

17   h (Fig. 1B) as was the case for B23.   On the other hand, the expression level of fibrillarin

18   involved in the processing of pre-rRNA and β-actin was almost equal between cells

19   maintained in 1% and 10% serum (Fig. 1B).   The reduction level of mRNAs of *UBF*,

20   *nucleolin*, *TIF-IA*, *fibrillarin*, and *β-actin* by low serum was virtually equal (see Fig. S1 in the

21   supplemental material).   These results suggest that the transcription by RNA polymerase II is

22   evenly affected by serum concentrations and the expression level of proteins involved in the

23   rRNA gene transcription is coordinately regulated at the level of translation and/or protein

1   degradation.   In parallel, the transcription rate of the rRNA gene was monitored by run-on

2   assays.   The transcription level of the rRNA gene in cells maintained in the presence of 1%

3   serum was lower by 0.7 fold than that in the presence of 10% serum (Fig. 1C).   The amount

4   of pre-rRNA was also monitored by quantitative RT-PCR using a primer set corresponding to

5   the 5' external transcribed spacer (5' ETS) of pre-rRNA (48) as described in Materials and

6   Methods in detail.   The results were normalized as a ratio to that of β-actin.   The amount of

7   pre-rRNA in cells maintained in the presence of 1% serum was lower by 0.6 fold than that in

8   the presence of 10% serum (Fig. 1D).   The number of rRNA transcripts in cells under the

9   growing condition is about 80% of the total cellular RNA quantity (40).   Thus, this

10   difference in the rRNA gene transcription rate between cells in 1% and 10% serum affect the

11   cellular metabolism significantly.   These results indicate that the rate of the rRNA gene

12   transcription and the cell growth are positively correlated with the expression level of genes

13   involved in the rRNA gene transcription including B23.   Hereafter, we focused functional

14   studies on B23.

15

16   **Knock-down of B23 decreases the transcription rate of the rRNA gene**

17   To investigate the role of B23.1 in transcription of the rRNA gene, knock-down

18   (KD) of the B23.1 expression was performed using short interfering RNA (siRNA) specific

19   for B23.1, siB23-A, -B, and -C.   Introduction of the siRNA decreased the protein level of

20   B23.1, but not β-actin, whereas a control siRNA had no effect (Fig. 2A).   The expression

21   level of other nucleolus-related genes was also examined in cells treated with siB23-C, and

22   siB23-C was found to have no effect on the expression of other nucleolus-related genes (Fig.

23   2B).   Therefore, we could examine the function of B23.1 without other possible indirect

1   effect of siB23-C.   KD of B23.1 was also confirmed in nucleoli of HeLa cells by

2   immunofluoresence using anti-B23 antibody (Fig. 2C).   The amount of pre-rRNA in HeLa

3   cells transfected with siB23 or control siRNA was examined by quantitative RT-PCR using 5'

4   ETS-specific primers for the rRNA gene.   The amount of pre-rRNA was normalized as a

5   ratio to that of β-actin mRNA.   KD of the B23 expression level resulted in decreased

6   transcription of the rRNA gene (Fig. 2D, right panel).   The trancription level of the rRNA

7   gene in cells treated by siB23-C was lower by 0.5 fold than that of control siRNA.   These

8   results indicate that B23 plays an important role in transcription of the rRNA gene by Pol I.

9

10   **B23 is associated with the chromatin around the rRNA gene**

11   The amino-terminal domain of B23 shows significant similarity to nucleoplasmin,

12   which is the first identified histone chaperone (32).   B23 has been shown to bind with

13   histones (45) and localized in nucleoli.   Thus, it is reasonably hypothesized that B23 binds to

14   chromatin around the rRNA gene.   To examine this, we performed nucleolar chromatin

15   immunoprecipitation (ChIP) as described in Materials and Methods.   Nucleoli isolated from

16   HeLa cells cross-linked with formaldehyde were sonicated to release chromatin fragments.

17   DNA purified from chromatin fragments ranged in size less than 1 Kbp (Fig. S3 in

18   supplemental material).   Sonicated chromatin was subjected to immunoprecipitation with

19   anti-Flag antibody as a negative control and anti-B23 antibody, followed by quantitative PCR

20   using a primer set specific for the rRNA gene promoter.   The antibody against TBP was also

21   used, since TBP is one of landmarks of the active promoter.   Figure 3A indicates that B23 as

22   well as TBP is associated with the chromatin around the rRNA gene promoter.   Next,

23   quantitative PCR was performed using primer sets corresponding to the entire rRNA gene

1 (Table 1) and immunoprecipitated chromatin DNA as a template. It is indicated that B23

2 binds the overall rRNA gene unit, but is concentrated at the transcription initiation site and the

3 region encoding 5.8S rRNA (Fig. 3B). Binding of B23 at the region encoding 5.8S rRNA

4 could correspond to the function of B23 for the processing of internal transcribed spacer 2

5 (ITS2) of pre-rRNA (22, 26, 51). The binding activity of B23 at the transcription initiation

6 site was examined in cells maintained in the presence of 1% or 10% of serum. The binding

7 activity of B23 to the rRNA gene in cells maintained in the presence of 10% serum is higher

8 than that in the presence of 1% (Fig. 3C). Moreover, we examined the chromatin structure

9 of the rRNA gene promoter region in cells treated with siB23-C. The amount of B23

10 associated with the rRNA gene promoter region was decreased in cells treated with siB23-C,

11 while that of fibrillarin or transcription factors of the rRNA gene, that are TBP and UBF, were

12 not influenced upon treatment with siB23-C (Fig. 3D, upper and middle panels). In contrast,

13 the amount of histone H3 associated with the rRNA gene increased upon treatment with

14 siB23-C (Fig. 3D, lower panel). The amount of histone H3 associated with the rRNA gene

15 chromatin was 2 fold higher in cells treated with siB23-C than that of control siRNA.

16 Histone distribution over the rRNA gene locus was examined (Fig. 3E, upper panel). The

17 amount of histone H3 associated with the rRNA gene in the cells treated with siB23-C was

18 higher than that of control cells over the rRNA gene locus. The ratio of the histone density

19 on the rRNA gene in cells treated with siB23-C relative to that in control cells is plotted (Fig.

20 3E, middle panel). This plot shows that the histone density around the transcription start site

21 and non-coding region between +15 Kbp and +20 Kbp relative to the transcription start site is

22 increased more than other regions. It is possible that the increase of the histone density on

23 the promoter region may decrease the transcription efficiency of the rRNA gene, although it is

1 presently unclear how the high histone density of the non-coding region affects the rRNA

2 gene transcription.   These results suggest that B23 is involved in control of the histone

3 density over the rRNA gene.

4

5 **B23ΔC inhibits the histone binding activity of B23.1 *in vitro***

6 To understand the function of B23 as a histone chaperone, we tried to prepare

7 deletion mutants of B23.1 aiming construction of a dominant-negative type mutant to block

8 the histone chaperone function of B23 specifically.   The acidic regions of B23 are crucial for

9 both histone binding and histone chaperone activities (44, 45).   The crystal structure of the

10 N-terminal domain of NO38, a *Xenopus* homolog of human B23, indicates that NO38 forms a

11 pentamer and each pentamer further assembles into a decamer as does nucleoplasmin (10, 41).

12 These studies suggest that oligomer formation of nucleoplasmin family proteins is important

13 for their histone chaperone activity.   Thus, we hypothesized that a B23 protein containing the

14 oligomeric domain but lacking domains responsible for its histone chaperone activity is to be

15 a dominant-negative type protein.   To test this, B23 mutants termed B23ΔA and B23ΔC

16 lacking either the acidic region or both the acidic and the carboxyl-terminal region,

17 respectively, was prepared (Fig. 4A).   Recombinant hexa histidine-tagged (His-) B23.1,

18 B23ΔA, and B23ΔC proteins were expressed in *E. coli* and purified (Fig. 4B).

19 To examine the histone binding activity of B23 mutants, histone binding assays

20 were carried out *in vitro* using recombinant B23 mutant proteins and core histones purified

21 from HeLa cells.   B23 proteins and core histones were mixed and incubated, followed by

22 immunoprecipitation with anti-His antibody and immunoblotting with anti-His and

23 anti-histone H3 antibodies.   His-B23ΔC did not bind to core histones, whereas the histone

17

1   binding activity of His-B23ΔA was essentially the same as that of wild type His-B23.1 (Fig.

2   4C).   The fact that B23ΔA binds to histone H3 indicates that the other domain except for

3   acidic domains is important for histone binding.   Nucleosome assembly activity of B23ΔA

4   was examined using supercoiling assay (Fig. 4D).   His-B23.1 assembled nucleosomes in a

5   dose-dependent manner, and His-B23ΔC did not as previously described (45).   Although

6   His-B23ΔA bound with core histones, it could not transfer core histones to plasmid DNA (Fig.

7   4D).   This result indicates that acidic domains of B23.1 are crucial for the histone chaperone

8   activity.

9   We confirmed that B23ΔC does not have histone binding and nucleosome assembly

10   activities (Figs. 4C and 4D).   Then, we examined whether B23ΔC is a dominant-negative

11   type protein for B23.1.   When mixed directly, His-B23ΔC did not form a hetero-oligomer

12   with His-B23.1 *in vitro*, suggesting that B23 forms a stable oligomer (data not shown).

13   However, when a mixture of His-B23.1 and His-B23ΔC was subjected to the

14   denature-renature protocol as described in Materials and Methods, two proteins were found to

15   form a hetero-oligomer successfully.   The interaction between B23 oligomers and core

16   histones was examined by immunoprecipitation using the anti-B23 antibody that can not

17   recognize B23ΔC (data not shown).   Immunoprecipitated proteins were separated by a

18   12.5% SDS-PAGE, followed by silver staining (Fig. 4E, left panel) and immunoblotting (Fig.

19   4E, right panel) using anti-His antibody.   B23.1 formed a hetero-oligomer with His-B23ΔC

20   (Fig. 4E, right panel, lanes 3 and 4).   B23.1 interacted efficiently with core histones (Fig. 4E,

21   left panel, lane 2) as shown in Fig. 4C and a previous report (45).   However, the histone

22   binding activity of B23.1 was decreased by oligomer formation with increasing amounts of

23   His-B23ΔC (Fig. 4E, left panel, lanes 3 and 4).   The molar ratio between His-B23.1 and

His-B23ΔC used here was 1:2.5 and 1:5 in lanes 3 and 4, 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 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, or Flag-B23ΔA

proteins (Fig. 5A). To examine the histone binding activity of B23 mutants,

immunoprecipitation with anti-HA and anti-Flag antibodies was performed using 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 the 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* (Figs. 5C and 5D). HA-B23.1 was expressed in 293T cells without

or with 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 rather

the amount of co-precipitated histone H3 with B23ΔA was slightly increased (Fig. 5C, lanes 8,

9, and 10). Then, Flag-B23.1 was expressed in 293T cells without or with 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 interacting 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 the 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 were co-precipitated with HA-B23ΔC under the condition 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 over-expression of HA-B23ΔC decreased this association by 25%. Next, we examined the effect of HA-B23ΔC on the chromatin structure

1   of the rRNA gene promoter region.   Binding of transcription factors of the rRNA gene, UBF

2   and TBP, was not affected or slightly up-regulated by expression of HA-B23ΔC (Fig. 6C),

3   while the amount of histone H3 or acetylated histone H3 associated with the rRNA gene

4   increased by expression of HA-B23ΔC (Fig. 6D).   Histone H3 distribution over the rRNA

5   gene locus was examined (Fig. 6E, upper panel).   The amount of histone in cells expressing

6   B23ΔC was higher than that in mock-treated cells over the rRNA gene locus.

7   Over-expression of B23ΔC increased the histone density of the coding region of 18S rRNA

8   gene (Fig. 6E, lower panel).   The histone density on the rRNA gene except for the region

9   encoding 18S rRNA is 1.3 ~ 1.8 fold higher in cells expressing B23ΔC compared with that of

10   mock-treated cells, consisting with the result using siB23 (Fig. 3E), whereas the histone

11   density on the region coding 18S rRNA was 2.9 fold higher in cells expressing B23ΔC than

12   that in mock-treated cells.   The reason for this discrepancy between the results in Fig. 3E and

13   Fig. 6E is presently unclear, but assumed to be due to the difference of cell types used in

14   experiments.   The results shown in Fig. 3D and Fig. 6D indicate that the amount ratio of

15   acetylated histone H3 to histone H3 on the rRNA gene in HeLa cells was lower than that of

16   293T cells.   In other words, this could be interpreted as that the ratio of the number of fully

17   active rRNA genes to the total number of the rRNA genes is higher in 293T cells than that of

18   HeLa cells.   Nevertheless, it is suggested that B23 controls the histone density on the rRNA

19   gene.

20   Next, we analyzed the transcription activity of Pol I in cells expressing HA-B23ΔC.

21   Nuclei were isolated from 293T cells expressing HA-B23ΔC, and run-on assays were carried

22   out (Fig. 6F).   Run-on assays demonstrated that the transcription level of the rRNA gene in

23   293T cells expressing HA-B23ΔC was lower by 0.6 fold than that of control cells, suggesting

21

1   that over-expression of HA-B23ΔC down-regulates the activity of Pol I possibly by blocking

2   the function of endogenous B23.   Moreover, we examined the effect of B23ΔA on the

3   transcription of the rRNA gene, which binds to histones but lacks the histone chaperone

4   activity (Fig. 4D).   Quantitative RT-PCR for pre-rRNA demonstrated that the amount of

5   pre-rRNA in cells expressing B23ΔA was lower than that of control cells (Fig. 6G).   This

6   result may be interpreted as that B23ΔA forms oligomer with endogenous B23.1 and inhibits

7   the rRNA transcription as does B23ΔC.   Thus, it is quite possible that B23 controls the

8   histone density on the rRNA gene, and thereby regulates the transcription of the rRNA gene as

9   a nucleolar histone chaperone.

10   We tried to confirm the above notion by complementation experiments.   By

11   monitoring the transcription of the rRNA gene, we examined the rescue ability of B23 mutant

12   proteins for HeLa cells treated with siRNA specific for B23.1.   After treatment of siB23-C,

13   HeLa cells were super-transfected with plasmids encoding various B23 proteins with

14   pEF321-T encoding SV40 T antigen in order to amplify the expression level of B23 proteins

15   supplied by pCAGGS and/or pCHA, which contain the replication origin of SV40.   siB23-C

16   recognizes the 3'UTR of endogenous mRNA of B23.1, and we constructed pCAGGS-based

17   plasmids encoding B23 mutant proteins without the siB23-C target sequence.   Therefore,

18   siB23-C does not affect the expression of exogenous B23 mutant proteins.   The expression

19   level of B23 proteins was detected by immunoblotting using anti-B23 or anti-HA antibodies

20   (Fig. 7A).   The amount of 45S pre-rRNA in HeLa cells was examined by quantitative

21   RT-PCR using the 5'ETS-specific primer set for the rRNA gene.   The reduction in synthesis

22   of 45S pre-rRNA by siB23-C was recovered by the expression of Flag-B23.1 in a

23   dose-dependent manner (Fig. 7B, lanes 2-4).   In contrast, Flag-B23ΔA and HA-B23ΔC could

1   not rescue the synthesis of 45S pre-rRNA reduced by siB23-C, but rather B23ΔC decreased

2   the synthesis of 45S pre-rRNA (Fig. 7B, lanes 2, and 5-8). These results indicate that

3   Flag-B23.1 promotes the transcription of the rRNA gene, but Flag-B23ΔA and HA-B23ΔC do

4   not. B23ΔA was shown to be capable of binding to histones (Fig. 4C), but it does not have

5   the histone chaperone activity (Fig. 4D), indicating that the acidic domain of B23.1 is critical

6   for the histone chaperone activity and the promoting activity of the rRNA gene transcription.

7   Thus, it is concluded that the histone binding activity of B23 is not sufficient for the rRNA

8   gene transcription promotion, and not only the histone binding activity but also the histone

9   chaperone activity is required (27).

10

11   **Inhibition of cell growth by over-expression of B23ΔC**

12   We have shown the positive correlation between the B23 histone chaperone activity

13   and the rRNA gene transcription. Next, we investigated the function of B23 in the cell

14   growth. To this end, we took advantage of B23ΔC, a dominant-negative type mutant for the

15   histone chaperone activity of B23. 293T cells were co-transfected with plasmids encoding

16   B23ΔC and EGFP. The number of EGFP-positive cells, thus quite possibly B23ΔC-positive

17   cells, was counted every 24 hrs post transfection (Fig. 8A). The cell growth of 293T cells

18   expressing B23ΔC seemed to be stopped. The cell growth rate was decreased in proportion

19   to the expression level of B23ΔC (Fig. 8B). In cells expressing B23ΔC3, the rRNA

20   synthesis was inhibited (Fig. 6). Moreover, KD of the expression of B23.1 by siB23-C

21   decreased the cell growth rate (data not shown). Thus, it is possible that B23ΔC suppresses

22   the synthesis of the rRNA gene, and thereby affects the cell growth rate.

23   Alternatively, B23ΔC may affect the cell growth through other pathway(s), since

1   B23 is a multifunctional protein. We examined the cell cycle profile of cells expressing

2   B23ΔC. It is suspected that the growth inhibition by expression of B23ΔC is due to

3   apoptosis. However, the population of apoptotic cells was not increased by expression of

4   B23ΔC (Fig. 8C, inlet table). As shown in Fig. 8C, G2/M population was slightly increased

5   by expression of B23ΔC. This result may be consistent with the observation with B23$^{-/-}$

6   MEFs (17, 31). Tetraploid cells contain 4n DNA as do cells in G2/M phase. On this line,

7   the p53 pathway involved in centrosome duplication that is essential for ploidy maintenance

8   is one of the candidates for the cell growth defect by expressing B23ΔC. It was reported that

9   B23 regulates the stability and the transcriptional activity of p53 directly or indirectly (5, 29).

10   To exclude the possibility that B23ΔC inhibited the cell growth through affecting the p53

11   pathway, we examined the effect of over-expression of B23ΔC on the cell growth rate of

12   p53$^{-/-}$ MEF (mouse embryonic fibroblast). As shown in Fig. 8D, the cell growth rate of

13   p53$^{-/-}$ MEF was similarly decreased by over-expression of B23ΔC. The effect of B23ΔC on

14   the cell growth of p53$^{-/-}$ MEF seems lower than that of 293T cells. This could be due to the

15   fact that a pCAGGS-based expression vector is amplified in 293T cells expressing SV40 T

16   antigen but not in p53$^{-/-}$ MEF, so that the expression level of B23ΔC is higher in 293T cells

17   than p53$^{-/-}$ MEF. Thus, the cell growth inhibition activity of B23ΔC is independent of the

18   p53 pathway. From these results, we concluded that over-expression of B23ΔC leads to the

19   growth inhibition most possibly through down-regulation of the rRNA gene transcription.

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 rRNA gene (Fig. 2). It was shown 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 (Figs. 4 and 5). B23ΔC over-expressed 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 neither B23ΔA nor B23ΔC could rescue the rRNA synthesis reduced by siRNA (Fig. 7). Finally, over-expression of B23ΔC results in inhibition of the cell growth (Fig. 8). These results altogether 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 ranged in almost the same level 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 under the transcriptionally repressive state. It is 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. Then, we would consider the possibility that

1 B23 is required for maintenance of an open chromatin. The results in this study show that

2 the rRNA gene transcription is proportional to the amount of B23. B23 remodels the

3 chromatin structure and stimulates the transcription from chromatin template (57, unpublished

4 results, and see below). We assume that the amount of B23 is a switch for the rRNA

5 transcription fluctuation in response to a variety of continuous stresses/stimuli from outside

6 and in side of cells.

7   In eukaryotic cells, the chromatin structure plays an important role in regulation of

8 gene expression. In the nucleolus, it was shown that NoRC, a nucleolar ATP-dependent

9 chromatin remodeling complex, recruits histone deacetylase and DNA methyltransferase

10 activities on the rRNA genes to induce formation of heterochromatin on the rRNA genes (38,

11 50). B23 has been shown to be acetylated by p300 and stimulate transcription preferentially

12 from a chromatin template containing acetylated histones, although the stimulation by

13 acetylated B23 occurs in transcription assay using RNA polymerase II in the presence of

14 transactivator Gal4-VP16 *in vitro* (57). This observation suggests that B23 facilitates

15 disassembly of histones over the rRNA gene in combination with p300 to stimulate the

16 transcription of the rRNA gene. Non-acetylable B23 mutants would be powerful tools for

17 further analysis of the coordination mechanism between B23 and p300 on the rRNA gene

18 transcription. In addition, B23 was shown to interact with nucleolin, which is one of

19 nucleolar histone chaperones and enhances the activity of the chromatin remodeling

20 machineries, SWI/SNF and ACF (1, 34, 48). B23 and nucleolin as nucleolar histone

21 chaperones may regulate the transcription of the rRNA gene through the structural change of

22 chromatin with ATP-dependent chromatin remodelers and histone modification enzymes in

23 coordinated manner.

1   Nucleoplasmin 3 (NPM3) was identified as a B23 binding protein from mouse liver

2   cDNA library using yeast two hybrid system (23).   NPM3 shows the significant structural

3   similarity to B23ΔC and is found to interact with endogenous B23.   Over-expression of

4   NPM3 decreased the rate of pre-rRNA synthesis and processing.   It is possible that NPM3

5   also functions as an inhibitor for the histone binding activity of endogenous B23 as does

6   B23ΔC.   We have also observed retardation of rRNA processing by overexpression of

7   B23ΔC (data not shown).   Since B23.2 lacking the C-terminal RNA binding domain was

8   found to form an oligomer with B23.1 and inhibit the RNA binding activity of B23.1 (46), it

9   is likely that B23ΔC also inhibits not only the histone chaperone activity but also the RNA

10   binding activity of B23.1.   The RNA binding activity of B23.1 could be important to target

11   B23.1 to pre-rRNA to be processed.   This could be one of the reasons why over-expression

12   of B23ΔC and NPM3 inhibits the pre-rRNA processing.

13   From the observation that B23, NPM3, and nucleolin are involved in pre-rRNA

14   synthesis and processing (1, 15, 23, 48, 51), it is likely that the rRNA gene transcription and

15   pre-rRNA processing are coupled and co-regulated.   This idea is supported by the result that

16   B23 associates with not only the promoter region but also the region encoding 5.8S rRNA

17   next to ITS2 cleaved by B23 (Fig. 3B).   In yeast cells, Pol I transcription and pre-rRNA

18   processing were shown to be linked by pre-rRNA processing factors (13, 16, 52) and the

19   complex containing Pol I drives both transcription and pre-rRNA modifications *in vitro* (11).

20   In mammalian cells, B23 could be one of the molecules to link between the rRNA gene

21   transcription and pre-rRNA processing.

22   We demonstrated that B23ΔC drastically decreased the cell proliferation rate of

23   293T cells (Fig. 8A).   B23$^{+/-}$, B23$^{hy/hy}$ (homozygous hypomorphic mutants) and B23$^{-/-}$ MEFs

1 show the decreased cell proliferation rate compared with wild type MEF (17).   It was

2 previously reported that B23 binds to p53 directly and enhances its stability and the

3 transcriptional activity of p53, so that over-expression of B23.1 induces inhibition of MEF

4 proliferation (5).   B23 interacts with ARF and Mdm2, those of which are involved in cell

5 cycle regulation in concert with p53 (3, 29, 30).   We had suspected that B23ΔC inhibits the

6 cell proliferation through the p53 pathway.   However, this possibility was ruled out by

7 experiments using p53$^{-/-}$ MEF (Fig. 8D).   This is supported by the report that a deletion

8 mutant of B23 similar to B23ΔC does not bind to p53 in vitro, and the expression of B23.1

9 increases the proliferation rate of p53$^{-/-}$ MEF slightly (5).

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1 **FIGURE LEGENDS**

2

3 **Figure 1**   Relationship between the cell growth and the level of rRNA gene transcription.

4 (**A**) Expression level of B23.   293T cells were maintained in DMEM in the

5 presence of 1% (lanes 1 and 3) or 10% (lanes 2 and 4) of FBS for 24 h (lanes 1 and 2) or 48 h

6 (lanes 3 and 4), and then pulse-labeled with [^{35}S]methionine for 1 h.   [^{35}S]-labeled B23

7 proteins were immunopurified using anti-B23 antibody and analyzed by electrophoresis on a

8 10% SDS-PAGE followed by autoradiography.   As a loading control, 10% of input lysates

9 was separated on a 10% SDS-PAGE, and β-actin was detected by immunoblotting.   (**B**)

10 Expression level of nucleolus-related genes.   293T cells were maintained in DMEM in the

11 presence of 1% (lanes 1 and 3) or 10% (lanes 2 and 4) of FBS for 24 h (lanes 1 and 2) or 48 h

12 (lanes 3 and 4).   Expression level of each indicated proteins were analyzed by

13 immunoblotting.   (**C**) Transcription rate of the rRNA gene.   293T cells were maintained in

14 DMEM in the presence of 1% or 10% of FBS for 72 h.   The expression level of the rRNA

15 gene was detected by run-on assays using [α-^{32}P]GTP.   Labeled rRNA was used as a probe

16 for hybridization to membrane-blotted plasmid DNAs containing the rRNA gene

17 (pUC-119-HurDNA) and pUC119 as a control.   Results are represented as mean values ± SD

18 from three independent experiments.   (**D**) Accumulation level of pre-rRNA.   293T cells

19 were maintained in DMEM in the presence of 1% or 10% of FBS for 72 h.   The expression

20 level of pre-rRNA and *β-actin* mRNA as a loading control was detected by quantitative

21 RT-PCR (left and middle panels).   The amount of pre-rRNA was normalized with that of

22 β-actin mRNA in the right panel.   Results are represented as mean values ± SD from three

23 independent experiments.

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Figure 2   Repression of the rRNA gene transcription by siB23

(**A**) Expression level of B23.1.   In the left panel, immunoblotting was performed using lysates prepared 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 (Fig. S2 in supplemental material).   (**B**) Expression level of nucleolus-related proteins.   The expression level of indicated each protein was determined by immunoblotting using HeLa cells treated with control siRNA and siB23-C.   (**C**) Expression of B23 in nucleoli.   Cellular B23 was stained by 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 position 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 (left panel) and primers for β-actin (middle panel).   The amount of pre-rRNA was normalized with that of β-actin mRNA (right panel).   Results are represented as mean values ± SD from three independent experiments.

Figure 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 ChIPed DNA as templates.   Results are represented as mean values ± SD from three independent experiments.   (**B**) ChIP analysis using Q-PCR through whole rRNA gene unit.

The structure of the human rRNA gene unit is shown under the graph. Grey boxes indicate 18S, 5.8S, and 28S rRNA encoding sequences. Horizontal arrow indicates the transcription initiation site (+1) of the rRNA gene transcribed by Pol I. DNA was extracted from soluble chromatin immunoprecipitated using anti-Flag and anti-B23 antibodies. Q-PCR was performed using ChIPed DNA as templates and primer sets indicated in Table 1. Results are represented as mean values ± SD from three independent experiments. (**C**) ChIP analysis using Q-PCR. 293T cells were maintained in DMEM in the presence of 1% or 10% of FBS for 72 h. DNA was extracted from chromatin that was immunoprecipitated using anti-Flag, and anti-B23 antibodies. Q-PCR was performed using promoter specific primers for the rRNA gene and ChIPed DNA as templates. Results are represented as mean values ± SD from three independent experiments. (**D**) Histone density on the rRNA gene promoter. Soluble chromatin fraction from HeLa cells treated with control siRNA or siB23-C was examined by ChIP analyses using anti-Flag, anti-B23, anti-UBF, anti-TBP, anti-acetylated histone H3, and anti-histone H3 antibodies. Q-PCR was performed using promoter-specific primers for the rRNA gene and ChIPed DNA as templates. Results are represented as mean values ± SD from three independent experiments. (**E**) Histone distribution over the rRNA gene. At the same time in (D), Q-PCR was carried out over the rRNA gene (upper panel). 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 is plotted (middle panel). Results are represented as mean values ± SD from three independent experiments.

Figure 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. Grey,

black, and striped boxes indicate 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 proteins (400 ng) were separated with a

12.5% SDS-PAGE followed by CBB 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 using an anti-His antibody.　Precipitated

proteins were analyzed by a 12.5% SDS-PAGE followed by immunoblotting with anti-His

and anti-histone H3 (α-H3) antibodies.　(**D**) Nucleosome assembly activity of B23 mutants.

The nucleosome assembly activity of B23 mutants was examined by supercoiling assay.

Core histones (200 ng) preincubated without (lane 1) or with His-B23.1 (167 ng for lane 2,

500 ng for lane 3, and 1500 ng for lane 4), His-B23ΔA (142 ng for lane 5, 425 ng for lane 6,

and 1275 ng for lane 7), and His-B23ΔC (66.8 ng for lane 8, 200 ng for lane 9, and 600 ng for

lane 10), 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), or 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.　Immunoprecipitation of His-B23.1 was carried out using

an anti-B23 antibody that can not recognize B23ΔC protein.　Precipitated proteins were

analyzed by a 12.5% SDS-PAGE followed by silver staining (left panel) and immunoblotting

1 (IB) with an anti-His antibody (right panel). H and L indicate heavy and light chains of

2 immunoglobulin, respectively. The position of core histones are indicated beside the left

3 panel.

4

5 **Figure 5** Inhibition of the histone binding activity of B23.1 by B23ΔC *in vivo*

6 (**A**) Expression of B23 proteins in 293T cells. Immunoblotting was carried out

7 using lysate prepared from 293T cells transfected with each plasmids indicated above each

8 lane. Left and right panels indicate immunoblotting by anti-HA and anti-Flag antibodies,

9 respectively. (**B**) Histone binding activity of B23 mutants. Immunoprecipitation (IP) with

10 anti-HA and anti-Flag antibodies were performed using lysate of 293T cells transfected with

11 plasmids expressing B23 proteins. Immunoprecipitated proteins were analyzed by

12 immunoblotting using anti-Flag, anti-HA, and anti-histone H3 antibodies, respectively. (**C**)

13 No effect of B23ΔA on the histone binding activity of B23.1. Lysates of 293T cells (24 h

14 post transfection) transfected with pCHA-B23.1 (160 ng for lanes 2-5, and 7-10) and

15 pCAGGS-Flag-B23ΔA (160 ng for lanes 3 and 8, 480 ng for lanes 4 and 9, and 1440 ng for

16 lanes 5 and 10), were subjected to immunoprecipitation with an anti-HA antibody.

17 Immunoprecipitated proteins were analyzed by immunoblotting using anti-Flag, anti-HA, and

18 anti-histone H3 antibodies, respectively. (**D**) Inhibition of the histone binding activity of

19 B23.1 by B23ΔC *in vivo*. Lysates of 293T cells (24 h post transfection) transfected with

20 pcDNA3-Flag-B23.1 (100 ng for lanes 2-4, and 6-8) and pCHA-B23ΔC   (300 ng for lanes 3

21 and 7, and 1400 ng for lanes 4 and 8), were subjected to immunoprecipitation with anti-Flag

22 antibody. Immunoprecipitated proteins were analyzed by immunoblotting using anti-Flag,

23 anti-HA, and anti-histone H3 antibodies, respectively.

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Figure 6   Inhibition of the 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 post transfection. Lysates prepared from 293T cells were used for immunoprecipitation with anti-HA antibody (lanes 3, 4, 7, and 8).   Immunoprecipitates (P) and flow-through fraction (F) were resolved by a 12.5% SDS-PAGE, transferred to a membrane, and probed with anti-B23 (upper panel) and anti-HA (lower panel) antibodies.   (**B**) Inhibition of association of B23 with the rRNA gene by B23ΔC.   293T cells were co-transfected with pBabe-puro and indicated plasmids (pCHA, pCHA-B23.1, and pCHA-B23ΔC), and 0.5 μg/ml puromycin was added after 24 h post transfection.   After puromycin selection for 30 h, 293T cells were maintained in fresh medium for 18 h.   Nucleolar ChIP was carried out using anti-B23 and anti-Flag antibodies. Quantitative PCR was performed with primers around the promoter region of the rRNA gene. Results are represented as mean values ± SD from three independent experiments.   A paired Student's *t*-test gave $P=0.02$ between pCHA- and pCHA-B23ΔC-transfected cells.   (**C**) Association of transcription factors on the rRNA gene promoter.   ChIP was carried out using anti-UBF and anti-TBP antibodies and lysates 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 represented as mean values ± 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 represented as mean values ± SD from three independent

43

experiments. (**E**) Histone distribution over the rRNA gene. ChIP was performed with anti-histone H3 antibody using cells expressing HA-B23ΔC or mock-treated cells.   Amount of ChIPed DNA was determined by Q-PCR using primer sets over the rRNA gene (upper panel).   The ratio of the histone density over the rRNA gene in cells expressing B23ΔC relative to that in mock-treated cells is plotted (middle panel).   Results are represented as mean values ± 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 post transfection.   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 represented as mean values ± SD from three independent experiments.   (**G**) Inhibition of the rRNA gene transcription by B23ΔA.   293T cells were co-transfected with pBabe-puro and either one of indicated plasmids (pCHA, pCHA-B23ΔC, and pCHA-B23ΔA), and 0.5 μg/ml puromycin was added after 24 h post transfection.   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 (left panel) and primers for *β-actin* mRNA (middle panel).   Results are represented as mean values ± SD from three independent experiments.

Figure 7   Involvement of acidic domains of B23 in the rRNA gene transcription

(A) Expression of B23 proteins in HeLa cells transfected with siB23.   HeLa cells

1 were transfected with 40 pmol of control siRNA (lane 1) and 40 pmol of siB23-C (lanes 2-8).

2 At 12 h after siRNAs transfection, HeLa cells were super-transfected with

3 pCAGGS-Flag-B23.1 (180 ng for lane 3, and 360 ng for lane 4), pCAGGS-Flag B23ΔA (180

4 ng for lane 5, and 360 ng for lane 6), pCHA-B23ΔC (180 ng for lane 7, and 360 ng for lane 8),

5 and pEF321-T (40 ng for lanes 1-8) encoding SV40 T antigen for amplification of transfected

6 plasmid.   After 72 h post siRNAs transfection, lysate of 2.5×10^3 cells were analyzed by

7 immunoblotting using anti-B23, anti-HA, and anti-β-actin antibodies, respectively.   (**B**)

8 Quantitative determination of pre-rRNA by Q-PCR.   At the same time with (A), total RNA

9 extracted from HeLa cells were examined by RT-PCR using 5'ETS-specific primers for the

10 rRNA gene.   The amount of pre-rRNA was normalized with that of β-actin mRNA.   Results

11 are represented as mean values ± SD from three independent experiments.

12

13 **Figure 8**   Inhibition of cell proliferation by B23ΔC

14   (**A**) Proliferation inhibition of 293T cells by expression of B23ΔC.   293T cells

15 were co-transfected with 1400 ng of pCHA and 200 ng of pEGFP-N1 or with 1400 ng of

16 pCHA- B23ΔCand 200 ng of pEGFP-N1.   The number of EGFP-positive cells was counted

17 every 24 hrs as a function of culture time.   Results are represented as mean values ± SD from

18 three independent experiments.   (**B**) B23 dose-dependent inhibition of the cell proliferation

19 rate.   The number of EGFP-positive cells was counted at 72 h after transfection of 200 ng of

20 pEGFP-N1 with pCHA-B23ΔC (350 ng (lane 2), 700 ng (lane 3), and 1400 ng (lane 4)), and

21 pCHA (1400 ng (lane 1), 700 ng (lane 2), and 350 ng (lane 3)).   Results are represented as

22 mean values ± SD from three independent experiments.   (**C**) Cell cycle population of cells

23 expressing B23ΔC.   293T cells were co-transfected with 1400 ng of pCHA and 200 ng of

1 pEGFP-N1 or with 1400 ng of pCHA-B23ΔC and 200 ng of pEGFP-N1, and then incubated

2 for 72 h.   Cell population of 293T cells expressing EGFP was determined by FACS analysis

3 (left panel).   Sub-G1 fraction was quantitatively determined as a population of apoptotic

4 cells and shown in the right inlet table.   (**D**) p53-independent effect of B23ΔC on the cell

5 proliferation.   The number of EGFP-positive p53$^{-/-}$ MEF cells was counted at 72 h post

6 transfection of pEGFP-N1 (125 ng) with pCHA-B23.1 (875 ng) or pCHA-B23ΔC (875 ng).

7 Results are represented as mean values ± SD from three independent experiments.

8