TITLE: B23/Nucleophosmin is involved in regulation of adenovirus chromatin structure at late infection stages, but not in its replication and transcription

RUNNING TITLE: Regulation of adenovirus chromatin by B23/nucleophosmin

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SUMMARY

B23/nucleophosmin was identified *in vitro* as a stimulatory factor for replication of adenovirus (Ad) DNA complexed with viral basic core proteins. In the present study, we have studied the *in vivo* function of B23 in Ad life cycle. We found that both the expression of a decoy mutant derived from Ad core protein V that tightly associates with B23 and siRNA-mediated depletion of B23 impede the production of progeny virions. However, B23 depletion did not significantly affect the replication and transcription of the virus genome. Chromatin immunoprecipitation analyses revealed that B23 depletion significantly increases the association of viral DNA with viral core proteins and cellular histones. These results suggest that B23 is involved in the regulation of association and/or dissociation of core proteins and cellular histones with the virus genome. In addition, our results suggest that proper viral chromatin assembly regulated in part by B23 is crucial for the maturation of infectious virus particles.

INTRODUCTION

Adenovirus (Ad) is an icosahedral particle with linear double-stranded DNA of approximately 36,000 base pairs in length. The linear DNA is covalently linked with virally coded terminal proteins and condensed with viral basic proteins Mu, VII, and V, thus forming a chromatin-like structure termed Ad core/Ad chromatin (Anderson *et al.*, 1989; Black & Center, 1979; Chatterjee *et al.*, 1985). Protein VII, a 19 kDa basic protein, is the major component of Ad core and most tightly associated with the Ad genome (Sung *et al.*, 1983). Protein V associates loosely with Ad DNA and forms an outer shell around Ad core to link it with the capsid through a dimer of polypeptide VI (Brown *et al.*, 1975; Chatterjee *et al.*, 1985; Fedor & Daniell, 1983). The virus genome is thought to be packed around the hexamer of core protein VII, and each unit of viral DNA-VII hexamer complex is bridged by core protein V (Dery *et al.*, 1985; Sung *et al.*, 1977).

Infecting virus particles are disassembled in the cytoplasm stepwise after penetration through endocytosis, and Ad core enters into the nucleus through nuclear pore complexes (Greber *et al.*, 1996; Martin-Fernandez *et al.*, 2004; Nakano *et al.*, 2000; Trotman *et al.*, 2001). During entry of the virus genome into the nucleus, core protein V seems to be dissociated from viral chromatin. Thus, viral DNA associated with core protein VII functions as a template for viral early gene transcription and DNA replication in the infected cell nucleus (Chatterjee *et al.*, 1986; Haruki *et al.*, 2006; Xue *et al.*, 2005). However, it was reported that core proteins function as a repressor for transcription and replication *in vitro* (Johnson *et al.*, 2004; Matsumoto *et al.*, 1995; Nakanishi *et al.*, 1986). Therefore, it is suggested that core proteins are either released or remodeled after entry into the host nucleus (Chen *et al.*, 2007; Matsumoto *et al.*, 1993; Matsumoto *et al.*, 1995; Spector, 2007). Histones may

associate with incoming viral DNA (Sergeant *et al.*, 1979; Tate & Philipson, 1979). Recently, it has been shown that not only protein VII but also cellular histones are functional components of viral chromatin in early phases of infection (Komatsu *et al.*, 2011). During late stages of infection, the precursor of core protein VII (pre-VII) and core protein V are synthesized at high levels concomitantly with viral DNA synthesis, assembled onto newly replicated DNA, and incorporated into immature virions (Daniell *et al.*, 1981). Newly replicated viral DNA may associate with cellular histones (Dery *et al.*, 1985). However, mature Ad particles do not contain cellular histones. It is still largely unknown how only the virus genome associated with viral core proteins is selectively incorporated into virions. It is also unknown which cellular factors are involved in this process.

Previously, we identified host factors termed Template Activating Factors (TAF)-I, -II, and -III from uninfected HeLa cell extracts that remodel the Ad core structure and stimulate replication and transcription from Ad core (Matsumoto *et al.*, 1993; Matsumoto *et al.*, 1995; Okuwaki *et al.*, 2001a). Recently, we have shown that TAF-I remodels the Ad core structure by forming a ternary complex with Ad DNA-core protein VII complexes and plays an important role in early stages of the Ad infection cycle (Gyurcsik *et al.*, 2006; Haruki *et al.*, 2003; Haruki *et al.*, 2006; Komatsu *et al.*, 2011). TAF-II is identical to nucleosome assembly protein-1 (NAP-1), a structural and functional homologue of TAF-I (Kawase *et al.*, 1996; Nagata *et al.*, 1995). The major component of TAF-III was found to be B23/nucleophosmin (Okuwaki *et al.*, 2001a).

B23/nucleophosmin is an abundant ubiquitously expressed cellular protein that modulates diverse molecular functions such as ribosome biogenesis (Hingorani *et al.*, 2000; Savkur & Olson, 1998), centrosome duplication (Okuda *et al.*, 2000),

chromatin assembly/disassembly (Okuwaki *et al.*, 2005; Okuwaki *et al.*, 2001b), and nucleo-cytoplasmic trafficking (Adachi *et al.*, 1993; Yu *et al.*, 2006). Two splicing variants of B23, B23.1 and B23.2, which differ only in their C-terminal regions, are expressed in a variety of growing cells. Both B23.1 and B23.2 contain highly acidic domains, while the C-terminal region unique for B23.1 is essential for its RNA binding activity. Recently, we have shown that B23 interacts with Ad core protein V, VII, and pre-VII, and may have a role as chaperone in the assembly of core proteins into Ad core (Samad *et al.*, 2007). However, an *in vivo* role(s) of B23 in the Ad life cycle has not been clarified yet. Here, we developed a decoy molecule for the interaction between B23 and core protein V based on the analysis of their interaction domains. Furthermore, we studied the effect of siRNA-mediated knock down (KD) of B23 on Ad proliferation. Perturbation of the B23 function either by over-expression of the decoy molecule or KD was shown to impede the Ad proliferation without significant inhibition of viral DNA replication or viral late gene expression. However, ChIP experiments indicated that the association of both core proteins and cellular histones with viral DNA was significantly increased upon B23 KD. Altogether, the results suggest that B23 is required for maintenance of the proper adenovirus chromatin structure.

RESULTS

Domains of core protein V required for its interaction with B23

Recently, we have shown that B23 interacts with Ad core proteins V and VII (Samad *et al.*, 2007). However, the function of B23 in Ad proliferation has not yet been clarified. To gain additional insight into the *in vivo* function of B23, we have designed a decoy molecule based on the analyses of interaction domains between B23 and core protein V. First, we determined the domain of core protein V required for the interaction with B23. Core protein V contains lysine- and arginine-rich basic clusters in its N- and C- terminal regions. We have postulated that core protein V interacts with B23 through these basic clusters, as it has been shown that the acidic region of B23 is essential for its function (Okuwaki *et al.*, 2001a). To test this hypothesis, we constructed a series of deletion mutants as shown in Fig. 1A. GFP- and Flag-tagged core protein V mutants were co-expressed with HA-tagged B23.1 in 293T cells, and immunoprecipitation assays were carried out with anti-Flag antibody. HA-B23.1 was co-immunoprecipitated with full length core protein V (Fig. 1B, lane 10). The mutants, V(1-313) and V(44-369), lacking the C- and N-terminal regions, respectively, similarly bound to HA-B23.1 (Fig. 1B, lanes 11 and 12). However, the mutants, V(44-313) and V(79-313), lacking both N- and C-terminal basic clusters showed virtually no ability to interact with HA-B23.1 (lanes 13 and 14). In contrast, the N- and C-terminal fragments, V(1-78) and V(314-369), respectively, efficiently co-precipitated HA-B23.1 (lanes 15 and 16). These results indicate that both N- and C-terminal regions are involved in the interaction between B23 and protein V, and these fragments are good candidates for decoy molecules for its interaction with B23.

Inhibition of infective virus production by B23 decoy molecule

We hypothesized that over-expression of these domains would interfere with the function of B23 in Ad proliferation if B23 is involved in Ad life cycle. To test this, HeLa cells were transfected with either GFP-empty vector or vectors for the expression of GFP-V, GFP-V(1-78), GFP-V(79-313), and GFP-V(314-369), and super-infected with HAdV5 at 20 hours after transfection. At 24 hpi, progeny virus particles were collected, and the infectivity titer was examined as described in Methods (Supplemental Fig. 1 and Fig. 1). Results demonstrate that the over-expression of GFP-core protein V, GFP-V(1-78), and GFP-V(314-369), but neither GFP alone nor GFP-V(79-313), inhibited the production of infectious virus particles. These results suggest that the mutant proteins that tightly associate with B23 inhibit the infectious virus production. It was further demonstrated that the co-expression of exogenous B23 with GFP-V(1-78) (Fig. 1D) rescued the negative effect of GFP-V(1-78). These results support the idea that V(1-78) functions as a sort of decoy for the interaction between B23 and core protein V, and the impairment of this interaction decreases the progeny virus production level.

B23 knock-down (KD) inhibits the production of infective viral particle

To further show that B23 is involved in Ad proliferation, we decreased the cellular B23 level using siRNA specific for B23.1. Although both B23.1 and B23.2 have been suggested to be differentially involved in Ad replication (Hindley *et al.*, 2007), we focused on B23.1 as it is concentrated in the nucleoli in which core protein V is located at the late stage of infection (Matthews, 2001), while B23.2 is distributed throughout the nuclei. In addition, the depletion of B23.1 alone efficiently decreased the nucleolar function of B23 (Murano *et al.*, 2008). Treatment of HeLa cells with B23.1 siRNA decreased the cellular B23.1 protein level but not β-actin, whereas

control siRNA had no effect (Fig. 2A). The expression level of nucleolar proteins such as nucleolin and fibrillarin were found to be unchanged upon B23 knock down (KD) (Fig. 2B). To examine the effect of B23 KD on Ad proliferation, control siRNA- and B23 siRNA-treated HeLa cells were infected with HAdV5. We first examined the effect of siRNA treatment on the localization of viral proteins (Fig. 2C). In control siRNA-treated cells, DBP was concentrated in nuclear foci and core protein VII distributed throughout nuclei at 24 hpi. As previously reported (Hindley *et al.*, 2007; Matthews, 2001), the nucleolar localization of B23 was slightly suppressed upon Ad infection, and B23 was partially co-localized with core protein VII but not with DBP. We also demonstrated that the localization patterns of DBP and core protein VII were not significantly affected by B23.1 siRNA treatment. At 24 hpi, the supernatant fraction containing progeny virus particles were collected, cleared by low speed centrifugation, and then examined for the infectious titer (Fig.2D). The production of infectious progeny virus particles from B23 KD cells were decreased to approximately 50 to 60% of that from control siRNA-treated cells (Fig. 2D, lanes 1 and 2). Even under the decreased B23.1 level, Ad virus production was increased until 48 hpi, although the amounts of produced infective virus during 24-36 hpi were lower than those of control siRNA-treated cells (Fig. 2C and supplemental Fig. 2). This result suggests that B23.1 is not essential but plays a crucial role in Ad virus production, and/or other cellular factor(s) could also be involved (see Discussion). Next, we investigated whether the effect of B23 KD on Ad virus production could be rescued by B23 over-expression. At 36 hours after introduction of control or B23 siRNA, cells were transfected with either empty vector or vector encoding HA-B23.1. Cells were then infected with Ad at 24 hours after transfection of plasmid DNA, and the production of progeny virus particles was examined. The expression level of

exogenous HA-B23.1 is shown in Fig. 2F. Over-expression of B23.1 in control cells slightly inhibited the infectious progeny virus production (Fig. 2D), although this result was not statistically significant. Importantly, the exogenous expression of B23.1 counteracted the negative effect of B23 siRNA-mediated KD on the progeny virus production. These results support the idea that B23.1 plays an important role in the production of infectious virus particles.

B23 KD has no significant effect on viral DNA replication and late gene expression

Given that B23 KD decreased the production of infectious virus particles, it was highly expected that this inhibition might be due to the interference with viral DNA replication. To test this notion, control or B23 siRNA-treated HeLa cells were infected with Ad and the amplification of viral DNA at 12, 18, and 24 hpi was examined by quantitative PCR using a primer set specific for Ad DNA (Fig. 3A). Because the amount of Ad DNA was increased as a function of incubation periods after infection and the amplification of DNA is strongly inhibited by hydroxyl urea (HU), it is confirmed that the PCR products detected under the condition employed here correspond to the amounts of the viral DNA. Surprisingly, no significant decrease in the amount of viral DNA upon B23 KD was observed (Fig. 3A). We also examined the effect of B23 KD on the late gene expression. The expression level of the late gene was examined by western blotting with anti-pVII and anti-V antibodies (Fig. 3B) and RT-PCR with primer sets for mRNAs of the major late promoter and pVII (Figs. 3C and D). Consistent with the fact that the late gene transcription depends on viral DNA replication, the expression level of late genes was strongly inhibited by the presence of HU. We did not find any significant decrease in both

mRNA and core proteins expression levels upon B23 KD (Figs. 3B-D). These results indicated that B23 plays a crucial role(s) in progeny virus production at a step(s) later than virus genome DNA replication and mRNA synthesis. Since Ad genome replication completely depends on viral early gene products, we could exclude the possibility that B23 is involved in the early gene transcription.

B23 regulates the amounts of core proteins and cellular histones on the Ad genome

Our biochemical data suggested that the Ad core proteins forms aggregates with viral DNA when mixed directly, and B23 dissociates the aggregation between DNA and core proteins (Samad *et al.*, 2007). In addition, we demonstrated that B23, as a histone chaperone, regulates the histone density around the rRNA gene region in uninfected cells (Hisaoka *et al.*, 2010). Therefore, it is possible that B23 KD affects the virus genome chromatin structure in infected cells. To test this possibility, we examined whether B23 is associated with the virus genome in infected cells. HeLa cells were infected with Ad, and at 20 hpi cells were cross-linked with formaldehyde and then sonicated to release chromatin. The average size of DNA purified from chromatin fragments was <1 kb (data not shown). The extracts were subjected to immunoprecipitation with antibodies against core proteins V, VII, or B23. We found that B23 associates with the virus genome (the VA gene region) as do core proteins V and VII (Fig. 4A). We examined the association of B23 with the virus genome using primer sets as shown in the bottom panel of Fig. 4B. Next, we assessed the amounts of core proteins and cellular histones on the virus genome with ChIP assays using cells treated with control and B23 siRNAs. HeLa cells treated with siRNAs were infected with HAdV5 at an MOI of 10. At 20 hpi, cells were subjected to ChIP assays

as described above. Five different primer sets as shown in Fig. 4B were used to examine the amounts of core proteins and histones on the Ad genome. In B23 KD cells, the association of both core proteins V and VII with viral DNA were found to be increased in all regions tested (Figs. 4C and D). We also found that the association of histone H3 along the virus genome is increased (Fig. 4E).

We also examined whether the amounts of core proteins and histone H3 on the virus genome increased by B23.1 KD were counteracted by exogenously expressed B23.1. HeLa cells treated with control or B23.1 siRNA were transiently transfected with empty and HA-B23.1 expression vectors, and then infected with Ad. At 24 hours after Ad infection, ChIP assays were carried out as shown in Figure 4 using primer sets specific for VA gene region (Figure 5). Western blotting analyses demonstrated that the amount of B23.1 was decreased efficiently by siRNA treatment, and that was recovered by transient expression of exogenous HA-tagged B23.1 (Figure 5A, lanes 5 and 6). Consistent with the data in Figure 4, the amounts of core proteins V, VII, and histone H3 on the virus genome was increased by B23.1 KD (Figure 5B-D). However, those were decreased upon over-expression of HA-B23.1. Interestingly, even in control siRNA-treated cells, HA-B23.1 over-expression decreased the association level of core protein V and histone H3. Taken together, these results suggest that B23 is involved in the regulation of viral chromatin formation in infected cells by restricting the access of core proteins and cellular histones.

DISCUSSION

In this paper, we have studied the *in vivo* function of B23 in Ad life cycle. Based on previous reports, it was expected that B23 may be involved in Ad DNA replication (Hindley *et al.*, 2007; Okuwaki *et al.*, 2001a). However, we could not detect any significant decrease in the amount of viral DNA (Fig. 3A) as well as the level of both transcription and translation of core proteins (Figs. 3B-D) upon B23.1 KD. Therefore, it is possible that B23.1 is not involved in Ad DNA replication in infected cells under the conditions employed here or the loss of B23.1 may be compensated alternatively. In this sense, it is noted that not only B23 but also other histone chaperones are identified as factors for Ad DNA replication (Kawase *et al.*, 1996; Matsumoto *et al.*, 1993; Okuwaki *et al.*, 2001a). In addition, we could not exclude the possibility that B23.2 remained in B23.1 KD cells plays a compensatory role in Ad DNA replication. Nevertheless, the data presented here demonstrate that B23.1 KD did not significantly affect DNA replication and transcription.

We have shown that the decrease of B23 reduces the production of progeny virions and increases the association level of viral core proteins and cellular histones on the progeny virus genome DNA. Based on these observations, we would propose that (1) B23 is involved in the adenovirus infection cycle at a step later than late gene expression, and (2) proper virus chromatin assembly is required for the Ad virion maturation. The precise mechanism of how B23 regulates viral chromatin and is involved in the final maturation step of infective virus particles remains unclear. It is also possible that in addition to B23, other cellular factor(s) is involved in these processes. TAF-I is a candidate of such additional factors, because we have reported that TAF-I is also associated with pre-VII in the late phases of infection (Gyurcsik *et al.*, 2006). It is suspected that the viral DNA associated with appropriate amounts of

basic proteins is important for encapsidation. In fact, the Ad genome DNA is condensed into a core structure only by viral basic proteins within virons, although viral DNA is associated with histones throughout the infection cycle (Dery *et al.*, 1985; Levy & Noll, 1981). Thus, it is likely that those virus genomes associated with cellular histones are restricted and eliminated for encapsidation. For efficient encapsidation to occur, cellular histones must be replaced with viral basic proteins through an unknown pathway.

Newly replicated DNA is associated with histones, and this viral DNA-histone complex might be important for ongoing replication and transcription throughout the infection cycle. At later stages of infection, the synthesis of cellular DNA and histones is inhibited with the concomitant accumulation of a large pool of viral basic proteins. The virus genomic DNA associated with viral core proteins might become prominent through direct interaction of ongoing replicated DNA with viral basic proteins and/or replacement of histones on the replicated DNA with viral basic proteins. Based on these observations, B23 may be involved in the final encapsidation step either by replacing histones with core proteins or by restricting the access of excessive amounts of viral basic proteins/histones to viral DNA. These models are in agreement with earlier assumption (Dery *et al.*, 1985; Komatsu *et al.*, 2011).

METHODS

Cell culture and viruses

HeLa cells were maintained in minimal essential medium (MEM; Nissui) supplemented with 10% fetal bovine serum (FBS) at 37°C. 293T cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM; Nissui) containing 10% FBS. Human adenovirus type 5 (HAdV5) used in this study was amplified and purified as previously described (Haruki *et al.*, 2006).

Plasmid construction and transfection

Construction of plasmids for a series of protein V mutants is described in Supplemental Methods. pCHA-B23.1 was prepared as described (Okuwaki *et al.*, 2002). Transient transfection of each plasmid was performed by the calcium phosphate precipitation method into 293T cells and by Gene-Juice (Novagen) into HeLa cells.

Antibodies

The antibodies used in this study were as follows: mouse monoclonal antibody for B23 that recognizes endogenous B23.1 was purchased from Invitrogen. Polyclonal antibody that recognizes both B23.1 and B23.2 was generated in rabbits using B23ΔC2 expressed in E. coli (Okuwaki *et al.*, 2001a) as an antigen. Rabbit anti-core protein V and mouse anti-DBP antibodies were obtained from Dr. W.C. Russel as generous donation. Rat polyclonal anti-pre-VII (Haruki *et al.*, 2003), mouse monoclonal antibody for Flag-tag, rabbit monoclonal antibody for hemagglutinnin (HA)-tag, rabbit polyclonal antibody for histone H3, and mouse monoclonal antibody for β-actin were described elsewhere (Murano *et al.*, 2008).

Immunoprecipitation assays

293T cells transiently transfected with plasmids where indicated were lysed in 1 ml of IP buffer (50 mM Tris-HCl, pH7.9, 1 mM PMSF, 0.1% Triton X-100, and 1 mg/ml bovine serum albumin) containing 150 mM NaCl on ice for 10 min followed by extensive sonication. Cell extracts recovered by centrifugation were mixed with anti-Flag antibody, and incubated at 4°C for 3 hours. Then, protein A sepharose beads (10 μl of resin; GE health care) were added and further incubated for 1 hour with gentle agitation. The beads were washed three times with 0.5 ml of IP buffer. Immunoprecipitated proteins were eluted by an SDS sample buffer, boiled, separated on a 12.5% SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was subjected to western blotting analysis using anti-HA antibody.

Decoy assays

HeLa cells were transfected with either GFP-empty vector or the vector for the expression of GFP-V(1-78). At 20 hours post transfection, cells were super-infected with HAd5 at an MOI of 10. At 24 hpi, culture supernatant (5 μl) clarified by low speed centrifugation was used for infection of HeLa cells seeded on coverslips in 12 well-plates (1×10^5 cells/well) to determine the virus titer. After incubation for 1 hour, cells were supplemented with MEM containing 2% FBS and maintained at 37°C in a 5% CO_2 environment for additional 15 hours. Cells on cover slips were collected, washed with PBS (-), fixed with 3% paraformaldehyde for 10 min at room temperature, and stained with anti-DBP antibody. Cells were also counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and the infectious titers (% of infected

cells) was determined by counting DBP positive cells out of DAPI positive cells. This assay was carried out in doublet, and error bars indicate SD.

B23 knock down by siRNA

B23 Stealth RNAi (NPM1-HSS143154) and Stealth RNAi negative control (catalog no. 12935-200) were purchased from Invitrogen and introduced into HeLa cells with Lipofectamine ^{TM} RNAiMAX (Invitrogen) according to the manufacturer's protocol. At 24 hours post transfection, the medium was replaced, and cells were harvested after 60 hours post siRNA transfection. Total HeLa cell lysates were prepared, and proteins were separated through 10% SDS-PAGE and detected by western blotting. To examine the effect of B23 KD on the virus production, control and B23 siRNA-treated HeLa cells at 60 hours post transfection were infected with HAdV5 at an MOI of 10. After 24 hours, the culture medium was recovered and examined for virus titer as described above.

Immunnofluorescence assay

Indirect immunofluorescence assays were carried out essentially as described previously (Haruki *et al.*, 2006). Briefly, cells grown on cover slips (15 mm; Matsunami) were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (RT) and then treated with 0.5% NP-40 in PBS for 5 min at RT. After blocking with 5% nonfat milk in TBS-T, samples were subjected to immunofluorescence analyses using antibodies described above. Localization of the protein was visualized with the secondary antibodies (anti-rabbit IgG conjugated with AlexaFluor 488, anti-mouse IgG conjugated with AlexaFluor 568, and anti-rat IgG conjugated with AlexaFluor 568; Invitrogen). DNA was visualized by staining with

TO-PRO-3 iodide (Invitrogen). Labeled cells were observed with confocal laser scanning microscopy (LSM5 Exciter; Carl Zeiss) using argon laser (488 nm) and He/Ne laser (546 and 633 mm) lines.

Quantitative PCR

Control and B23 siRNA-treated HeLa cells were infected with HAdV5 at an MOI of 10. At 12, 18, and 24 hours post infection, cells (1×10^5) were collected and suspended in lysis buffer (20 mM Tris pH7.9, 100 mM NaCl, 5 mM EDTA, and 0.5% SDS), and total DNA was purified with proteinase K treatment at 50°C for overnight followed by phenol-chloroform extraction and ethanol precipitation. The amount of DNA was then examined by quantitative (q) PCR with a primer set specific for the Ad VA gene region (see below). Total RNA was purified from infected cells (1×10^5) using RNeasy mini kit (QIAGEN), and the purified RNA was treated with DNase I according to the manufacturer's protocol. The concentration of RNA in each sample was determined by using NanoDrop (Thermo Scientific). cDNA was synthesized from total RNA (1 μg) using ReverTraAce (Toyobo) and oligo-dT as primer according to the manufacturer's protocol. qPCR with FastStart SYBER Green Master Mix (Roche) and Thermal Cycler Dice Real Time System (Takara) was performed using synthesized cDNA as a template with primer sets specific for the mRNA from major late promoter (MLP), 5'-ACTCTCTTCCGCATCGCTGT-3' and 5'-GTGACTGGTTAGACGCCT-TTCT-3', and β-actin gene, 5'-ATGGGTCAGAAGGATTCCTATGT-3' and 5'-GGTCATCTTCTCGCGGTT-3'.

ChIP assays

ChIP assays were carried out according to the manual of ChIP assay kit (Millipore) with anti-core protein V, pVII, B23, and histone H3 antibodies. The amount of immunoprecipitated DNA was determined by qPCR as described above. The reaction condition was described previously (Komatsu *et al.*, 2011). The following primer sets were used; 5'-GGGTCAAAGTTGGCGTTTTA-3' and 5'-CAAAATGGCTAGGAGGTGGA-3' for the E1a promoter region, 5'-GCGGTCCTCCTCGTATAGAA-3' and 5'-CCCACCCCCTTTTATAGCC-3' for the ML promoter region, 5'-GCTGGAGCAAAACCCAAATA-3' and 5'-TATCTTGCGGG-CGTAAAACT-3' for the VA region, 5'-GTGTAGACACTTAAGCTCGCCTT-3' and CTTCAAACTGCCTGACCAAGT-3' for the E2A (DBP) region, and 5'-TGGCGTGGTCAAACTCTACA-3' and 5'-GATTTTTACAATGGCCGGACT-3' for the E4 ORF region.

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FIGURE LEGENDS

FIG. 1. N- and C-terminal regions of core protein V are required for the interaction with B23. (A) Schematic diagram of wild type and mutant protein V. GFP-Flag-tag was fused at the N-termini of core protein V and its mutant proteins. The basic clusters of core protein V are indicated by filled boxes. (B) Immunoprecipitation of Flag-core protein V. 293T cells were transiently co-transfected with HA-B23.1 and either GFP-Flag-V or its mutants. After immunoprecipitation without (-) or with (+) anti-Flag antibody, proteins in input extracts (lanes 1-8) and precipitated proteins (lanes 9-16) were separated through a 12.5% SDS-PAGE and detected by western blotting with anti-HA and anti-Flag (top and bottom panels, respectively) antibodies. Positions of GFP-Flag-tagged protein V and its mutants are indicated by arrow heads at the left side of each lane. **(**C) Inhibition of virion production by a decoy molecule. HeLa cells were transfected with either GFP-empty vector or a vector expressing GFP-Flag-tagged V(1-78) mutant. At 20 hours after transfection, cells were infected with HAd5. At 24 hpi, viruses in the culture fluid were collected and examined for virus titer. In the bottom panel, expression of exogenous proteins as well as β-actin was confirmed by western blotting. (D) Rescue experiments. Cells expressing either GFP-Flag (lane 1), GFP-Flag-V(1-78) with pCHA empty vector (lane 2), or GFP-Flag-V(1-78) with pCHA-B23.1 vector (lane 3). At 24 hour after transfection, cells were super-infected with Ad and virus production was examined by the procedure as described in (C). Exogenously expressed proteins were detected by western blotting as shown at the bottom of the panel.

FIG. 2. B23 is involved in Ad proliferation. (A) Knockdown (KD) of B23.1. HeLa cells were transfected with control siRNA (lane 4) or B23 siRNA (lane 5), and the expression level of B23 was examined by western blotting with anti-B23 antibody. HeLa cell lysates from 5×10^3, 1.5×10^4, and 5×10^4 cells for lanes 1, 2, and 3, respectively, were loaded on the same gel and used as standards. β-actin is shown as a loading control. (B) Expression of nucleolar proteins. The expression level of indicated proteins was determined by western blotting using HeLa cells treated with control siRNA or B23.1 siRNA (lanes 1 and 2, respectively) as in (A). (C) Localization of viral proteins in B23.1 siRNA-treated cells. HeLa cells treated with control- or B23.1-siRNA as indicated at the top of the panels were super-infected with Ad and subjected to indirect immunofluorescence analyses at 24 hpi. B23 and DBP (top panels) or B23 and core protein VII (bottom panels) were simultaneously stained and visualized. DNA was counter-stained with To-Pro-3. (D) Inhibition of infectious virus production by KD of B23. HeLa cells treated with either control- or B23.1-siRNA were infected with HAd5. At 24 hpi, virus titers in the culture medium were examined as described in Methods (lanes 1 and 2). At 36 hrs after siRNA transfection, pCHA empty vector (lanes 3 and 4) or pCHA-B23.1 (lanes 5 and 6) were transfected and incubated for 24 hrs. Then, cells were super-infected with HAd5, and the virus infectivity was determined at 24 hpi as described above. Experiments were carried out in triplicate, and error bars indicate standard deviations (SD). Statistical *P* values are indicated at the top of the graph. (E) Expression level of endogenous and exogenous B23. Lysates prepared as indicated in (D) were analyzed by western blotting with anti-B23.1 and anti β-actin antibodies (top and bottom panels, respectively).

FIG. 3. Effect of B23 KD on Ad DNA replication and late gene expression. (A) Effect of B23.1 KD on Ad DNA replication. Control or B23.1 siRNA-treated HeLa cells (black and white bars, respectively) were infected with Ad and incubated without (-) or with (+) 2 mM hydroxyl urea (HU) as indicated at the bottom of the panel. At 12, 18, and 24 hpi, DNA was purified from infected cells, and the amount of viral DNA was examined by quantitative PCR using a primer set specific for the VA region of the Ad genome. Genomic DNA purified from HeLa cells infected with Ad was used as standards for the amount of the Ad genome in infected cells, and the relative amounts of Ad DNA was normalized by that of β-actin gene. (B) Expression level of late gene products. Lysates were prepared from infected HeLa cells as described in (A) at 24 hpi. Proteins were separated on SDS-PAGE, and analyzed by western blotting with anti-β-actin, -B23, -core protein V, and -pVII antibodies. (C, D) Expression level of Ad late genes. HeLa cells treated with siRNA as described in A were infected with Ad. Total RNA was prepared at 18 hpi, and the expression level of MLP mRNA (C) and pVII mRNA (D) was determined by quantitative RT-PCR using specific primer sets as described in Methods. PCR reactions were performed in triplicate, and error bars indicate SD. Three independent experiments showed similar results.

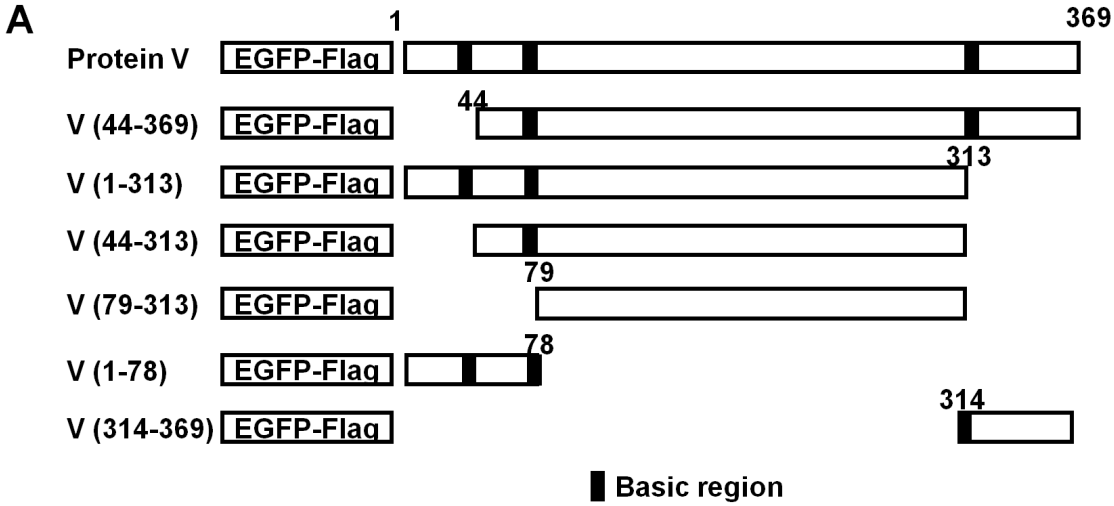
FIG. 4. Effect of B23 KD on the Ad viral chromatin structure. (A) ChIP assays were carried out with extracts prepared from Ad-infected HeLa cells at 20 hpi. DNA immunoprecipitated without (lane 2) or with anti-pVII, anti-V, and anti-B23 (lanes 3-5) antibodies was examined by PCR using a primer set specific for the Ad VA gene. (B) B23 associates with entire Ad genome in infected cells. Immunoprecipitated DNA with anti-B23 as described in (A) was subjected to quantitative PCR using

primer sets specific for Ad genome. Positions of primer sets used are schematically represented at the bottom of the panel. Arrows indicate the positions and direction of the transcription of each gene. DNA extracted from input extracts was used as standards to quantify the amount of DNA immunoprecipitated with anti-B23 antibody. (C-E) ChIP assay of Ad-infected HeLa cells treated with siRNAs. Control or B23.1 siRNA-treated HeLa cells were infected with Ad, and ChIP assays were carried out with anti-core protein V, anti-pVII, anti-histone H3 antibodies (C-E, respectively). Immunoprecipitated DNA was quantitatively examined by q-PCR using primer sets shown in (B). Black and white bars in graphs indicate the results obtained from extracts prepared from control and B23.1 siRNA-treated HeLa cells, respectively. The amounts of immunoprecipitated DNA were quantitatively analyzed compared with those of DNAs extracted from input extracts. For B-E, PCR reaction was carried out in triplicate, and error bars indicate SD. Two independent experiments showed similar results.

FIG. 5. Over-expression of exogenous B23.1 counteracts the effect of B23.1 KD on viral chromatin structure. (A) The expression levels of endogenous and exogenous B23.1. HeLa cells were treated with control- or B23.1-siRNA (lanes 1, 3, and 5 or 2, 4, and 6) without or with transfection of pCHA (lanes 3 and 4) or pCHA-B23.1 (lanes 5 and 6), and then infected with Ad. The expression level of B23 and β-actin was examined by western blotting analyses. (B-D) ChIP assays. HeLa cells prepared as described in (A) were subjected to ChIP assays with anti-core protein V (B), -core protein VII (C), or histone H3 (D) antibody. Immunoprecipitated DNA was quantitatively examined by q-PCR using primer sets specific for the Ad VA gene region. Black and white bars in graphs indicate the results obtained from extracts

prepared from control and B23.1 siRNA-treated HeLa cells, respectively. The amount of immunoprecipitated DNA was quantitatively analyzed compared with those of DNAs extracted from input extracts. PCR reactions were carried out in triplicate, and error bars indicate SD.

Samad Fig. 1


A
1
369
Protein V
EGFP-Flag
44
V (44-369)
EGFP-Flag
313
V (1-313)
EGFP-Flag
V (44-313)
EGFP-Flag
79
V (79-313)
EGFP-Flag
78
V (1-78)
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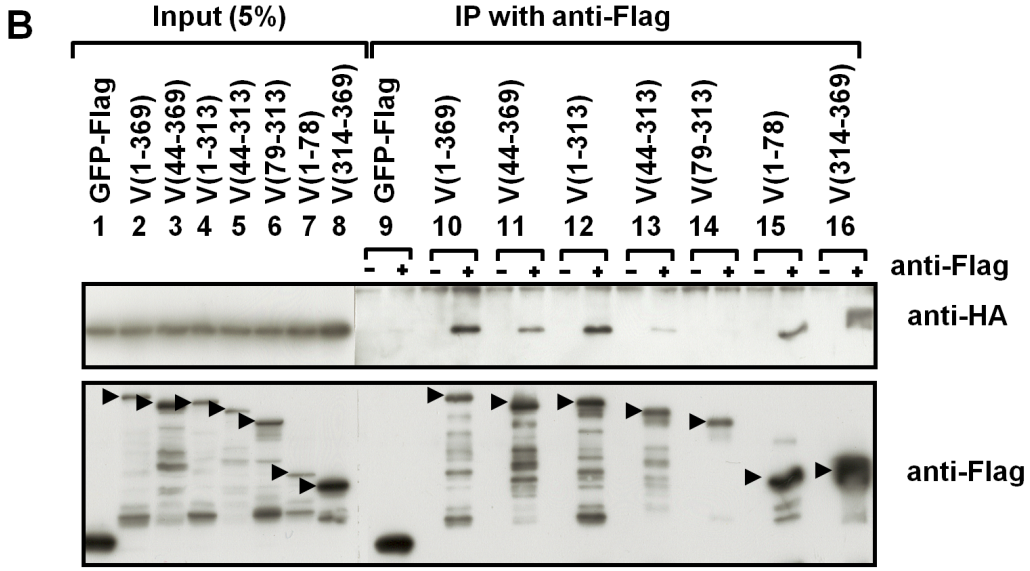
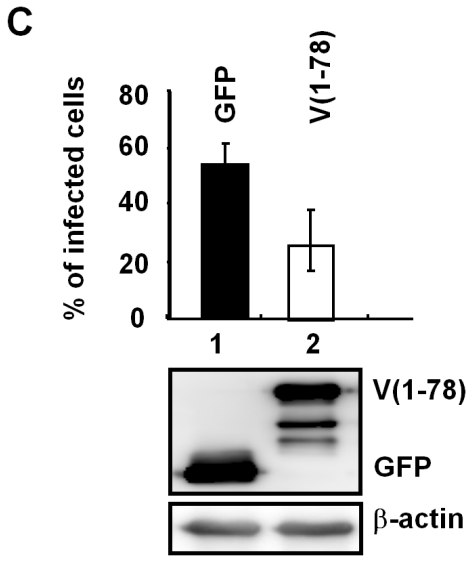
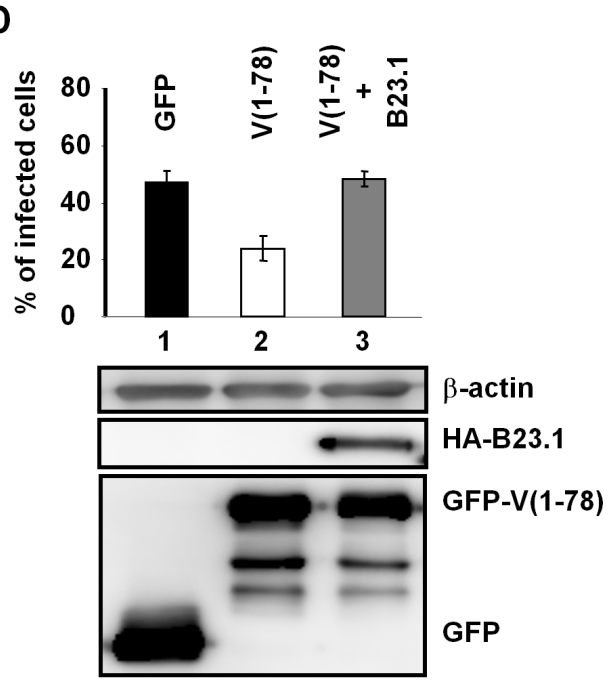
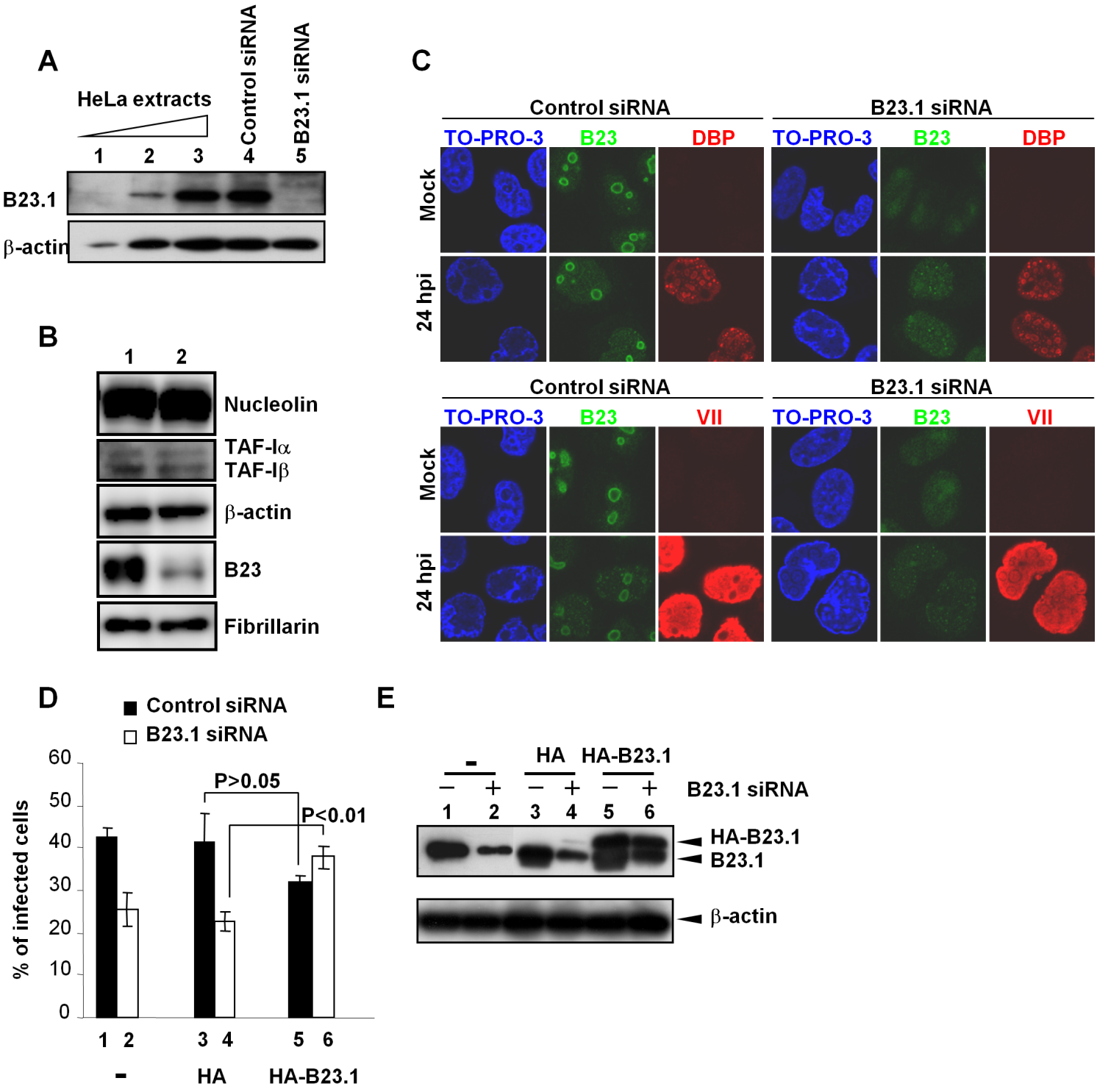
V (314-369)
EGFP-Flag
Basic region


B
Input (5%)
IP with anti-Flag
GFP-Flag
V(1-369)
V(44-369)
V(1-313)
V(44-313)
V(79-313)
V(1-78)
V(314-369)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
anti-Flag
anti-HA
anti-Flag

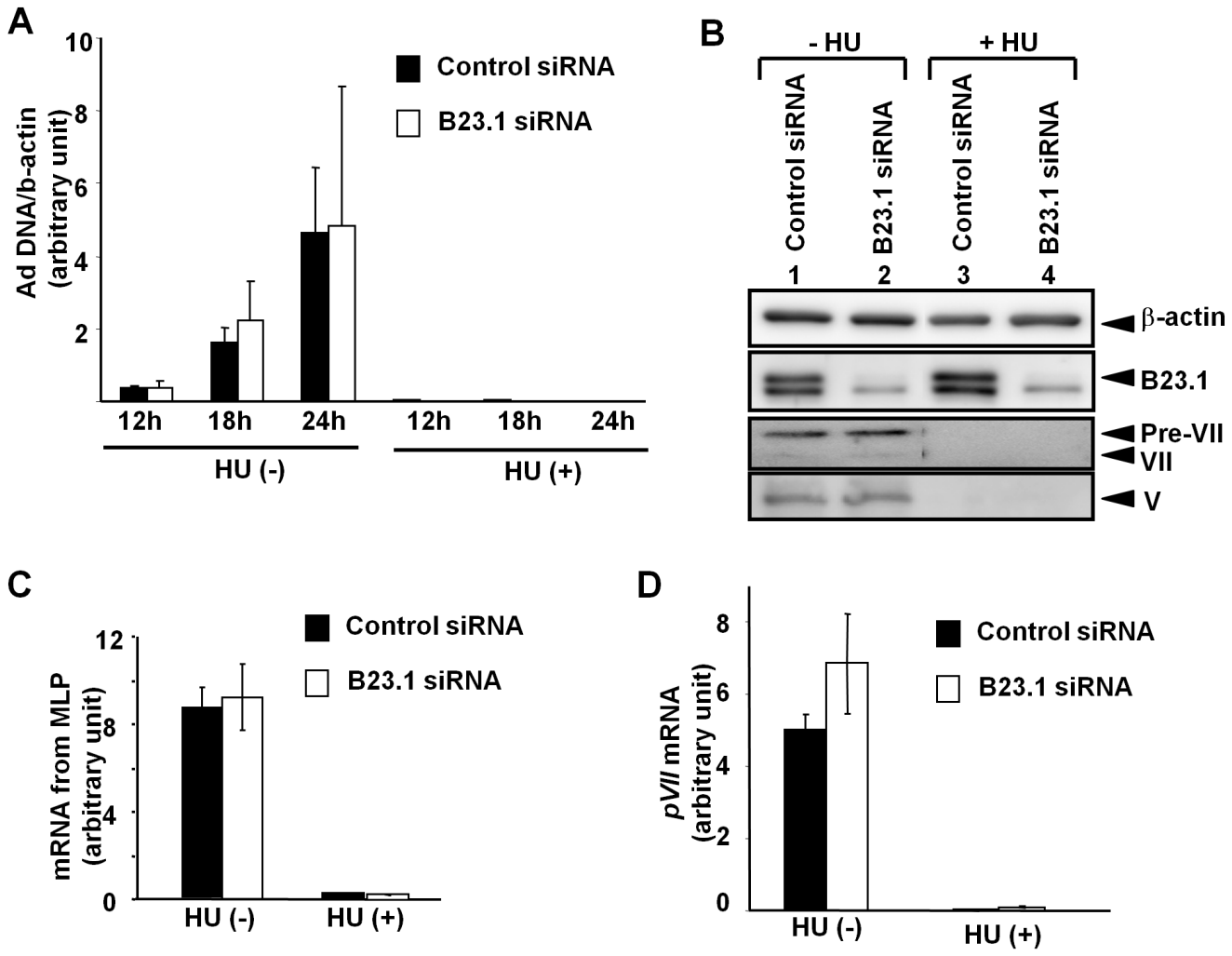

C
% of infected cells
80
60
40
20
0
GFP
V(1-78)
1
2
V(1-78)
GFP
β-actin


D
% of infected cells
80
60
40
20
0
GFP
V(1-78)
V(1-78) + B23.1
1
2
3
β-actin
HA-B23.1
GFP-V(1-78)
GFP

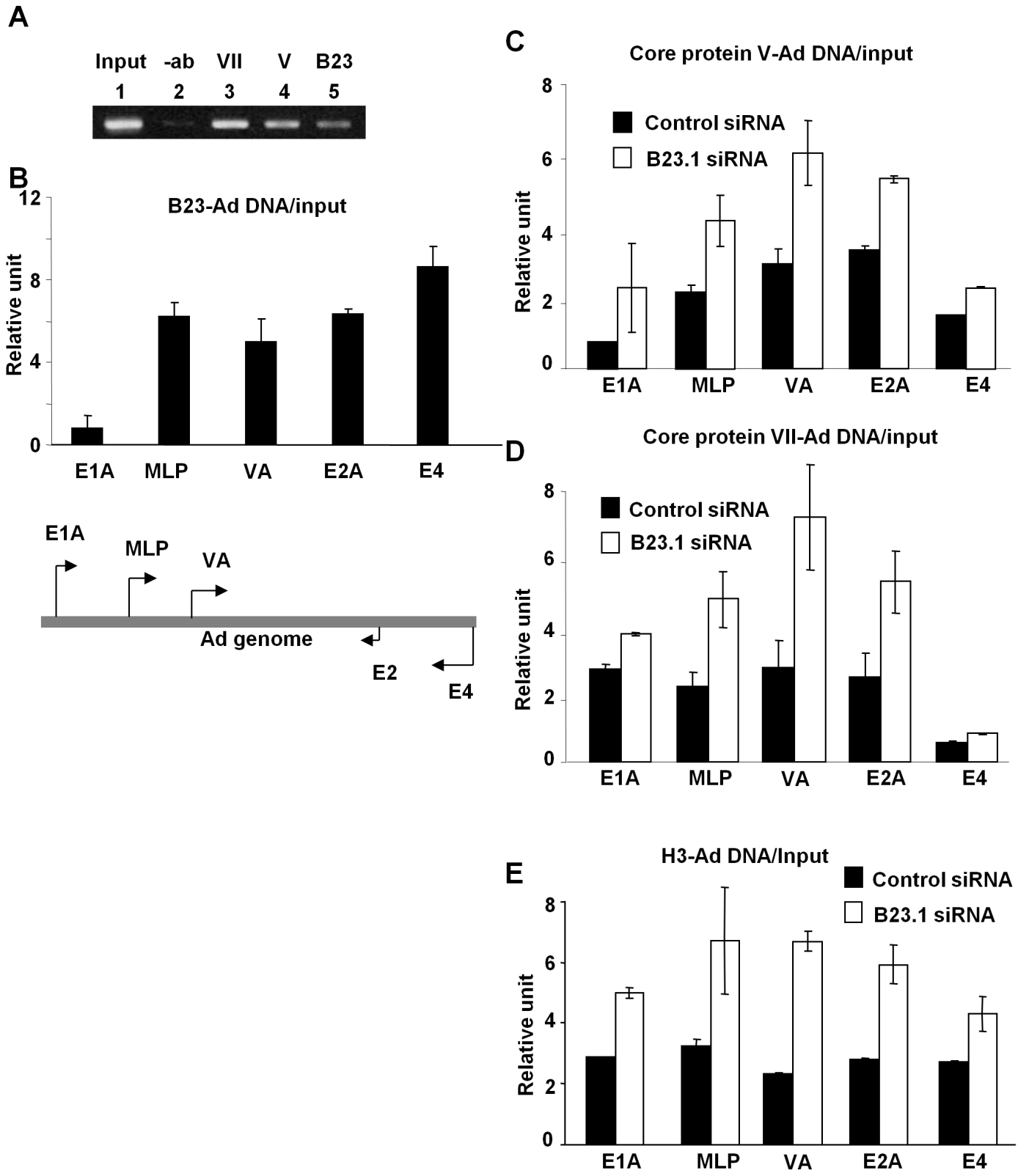
Samad Fig. 2


A
HeLa extracts
1 2 3
4 Control siRNA
5 B23.1 siRNA
B23.1
β-actin
B
1 2
Nucleolin
TAF-Iα
TAF-Iβ
β-actin
B23
Fibrillarin
C
Control siRNA
B23.1 siRNA
TO-PRO-3
B23
DBP
VII
Mock
24 hpi
D
Control siRNA
B23.1 siRNA
% of infected cells
60
50
40
30
20
10
0
P>0.05
P<0.01
1 2 3 4 5 6
-
HA
HA-B23.1
E
-
HA
HA-B23.1
B23.1 siRNA
1 2 3 4 5 6
HA-B23.1
B23.1
β-actin

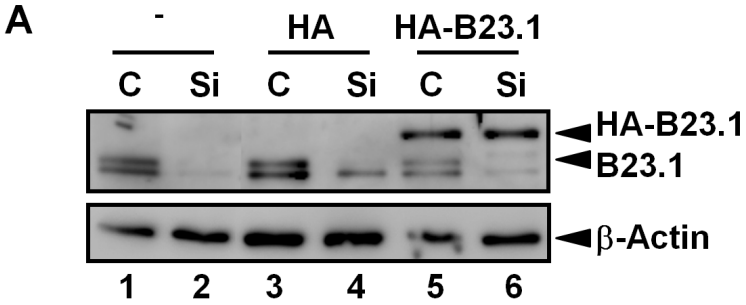
Samad Fig. 3

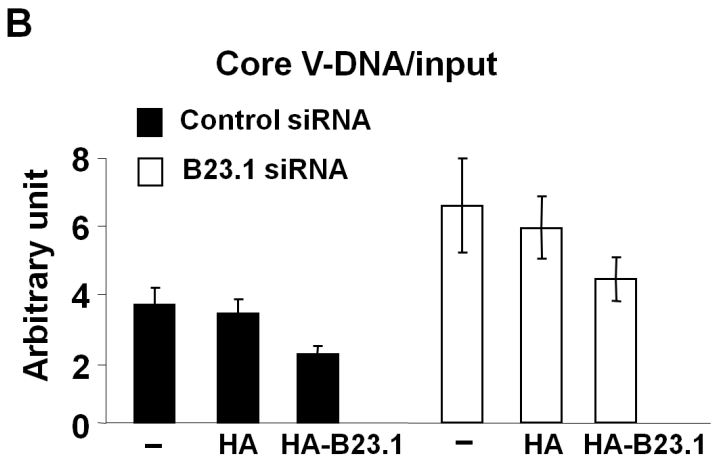

A
Ad DNA/b-actin (arbitrary unit)
10
8
6
4
2
Control siRNA
B23.1 siRNA
12h
18h
24h
12h
18h
24h
HU (-)
HU (+)
B
- HU
+ HU
Control siRNA
B23.1 siRNA
Control siRNA
B23.1 siRNA
1
2
3
4
β-actin
B23.1
Pre-VII
VII
V
C
mRNA from MLP (arbitrary unit)
12
8
4
0
Control siRNA
B23.1 siRNA
HU (-)
HU (+)
D
pVII mRNA (arbitrary unit)
8
6
4
2
0
Control siRNA
B23.1 siRNA
HU (-)
HU (+)

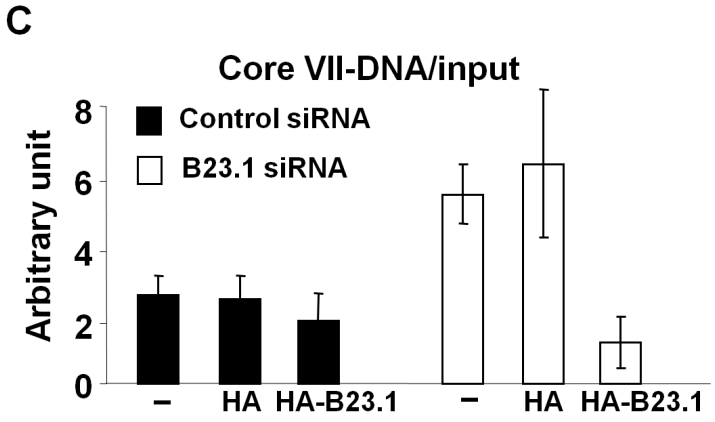
Samad Fig. 4


A
Input -ab VII V B23
1 2 3 4 5
B
B23-Ad DNA/input
Relative unit
12
8
4
0
E1A MLP VA E2A E4
E1A
MLP
VA
Ad genome
E2
E4
C
Core protein V-Ad DNA/input
Control siRNA
B23.1 siRNA
Relative unit
8
6
4
2
0
E1A MLP VA E2A E4
D
Core protein VII-Ad DNA/input
Control siRNA
B23.1 siRNA
Relative unit
8
6
4
2
0
E1A MLP VA E2A E4
E
H3-Ad DNA/Input
Control siRNA
B23.1 siRNA
Relative unit
8
6
4
2
0
E1A MLP VA E2A E4

Samad Fig. 5


A
-
HA
HA-B23.1
C
Si
C
Si
C
Si
HA-B23.1
B23.1
β-Actin
1
2
3
4
5
6


B
Core V-DNA/input
Control siRNA
B23.1 siRNA
Arbitrary unit
0
2
4
6
8
–
HA
HA-B23.1
–
HA
HA-B23.1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Core VII-DNA/input
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B23.1 siRNA
Arbitrary unit
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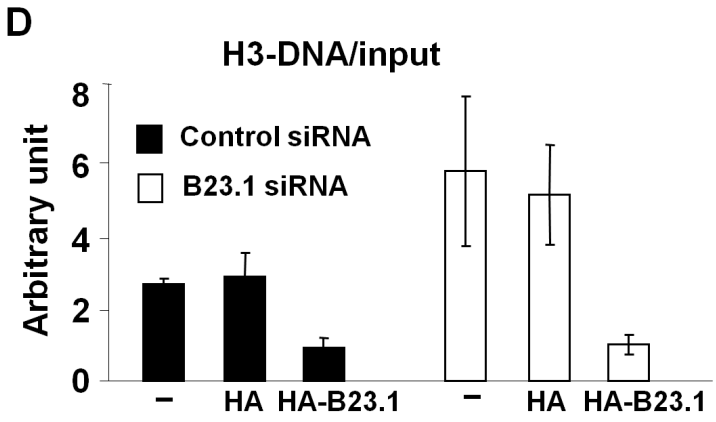
8
–
HA
HA-B23.1
–
HA
HA-B23.1


D
H3-DNA/input
Control siRNA
B23.1 siRNA
Arbitrary unit
0
2
4
6
8
–
HA
HA-B23.1
–
HA
HA-B23.1