1 Title: Physical and functional interaction between a nucleolar protein nucleophosmin/B23 and
2 adenovirus basic core proteins.
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Abstract

We identified nucleophosmin/B23 as a component of Template Activating Factor-III that stimulates the DNA replication from the adenovirus DNA complexed with viral basic core proteins. Here, we have studied the functional interaction of B23 with viral core proteins. We found that B23 interacts with viral basic core proteins, core protein V and precursor of core protein VII (pre-VII), in infected cells. Biochemical analyses demonstrated that B23 suppresses formation of aggregates between DNA and core proteins and transfers pre-VII to DNA. These results indicate that B23 functions as a chaperone in the viral chromatin assembly process in infected cells.

Key words: Adenovirus, B23, Chromatin, Core protein, Nucleolus

1. Introduction

The adenovirus (Ad) genome is a linear double-stranded DNA of approximately 36,000 base pairs (bp) in length, and packed into the non-enveloped icosahedral particle. The viral DNA is covalently linked with virally encoded terminal protein and condensed in the virion with viral core protein VII, core protein V, and polypeptide X, thus forming a chromatin-like structure, hereafter designated as Ad core [1-3]. Core protein VII, a 19 kDa protein with limited amino acid sequence similarity to histone H3 and a basic sperm-specific protein, is a major component of Ad core and tightly associated with the Ad genome [4]. Core protein V associates loosely with Ad DNA and forms an outer shell around Ad core to link it with the capsid by binding with a dimer of polypeptide VI [5-7]. The infecting virus disassembles gradually in the cytoplasm after entry and penetration into host cells [8-11]. Core protein V is removed from Ad core immediately after entry into the nucleus, whereas core protein VII remains associated with the genome [12,13]. Therefore, transcription of the early genes and the first round of viral DNA replication start using Ad DNA complexed with core protein VII as a template in infected cells. During late stages of infection, the precursor of core protein VII (pre-VII) and core protein V package the newly replicated DNA into Ad core. The detail molecular mechanism of disassembly and assembly of Ad core is not clear at present.

We identified nucleophosmin/B23 as a component of Template Activating Factor (TAF)-III that stimulates the DNA replication from Ad core [14]. 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, and the C-terminal region unique for B23.1 is essential for its RNA binding activity. The nucleolar localization of B23 is disrupted upon Ad infection, and core protein V was shown to be responsible for re-localization of B23 from the nucleolus to the whole cell [15]. Thus, it is possible that B23 is involved in the Ad life cycle. However, the functional interaction between B23 and factors associated with Ad infection has not been described yet.

To explore the function of B23 in the Ad life cycle, we have studied the interaction between B23 and Ad core proteins. We found that B23 interacts with both viral core protein V and pre-VII during late stages of infection. Biochemical analyses demonstrated that B23 can induce dissociation of core proteins from DNA-core protein aggregates. In addition, B23 was found to mediate formation of nucleoprotein complexes containing pre-VII. Taken together, it is indicated that B23 functions as a chaperone for viral chromatin assembly.

2. Materials and methods

2.1. Plasmid construction and protein purification

cDNA corresponding to the protein V was amplified by polymerase chain reaction (PCR) from the human adenovirus type 5 (HAdV-5) genomic DNA using a primer set, 5'-AAGCTCGCATATGTCCAAGCGCAAAATCAAA-3' and 5'-AAGCTAAGGATCCTTAAACGATGCTGGGGTGGTA-3'. The amplified PCR product was cloned into *Nde*I- and *Bam*HI-digested pET14b (Novagen). His-protein V expressed in the *E.coli.* strain BL21 codon plus RIL (STRATAGENE) was purified using His-bind resins (Novagen) according to the manufacturer's instruction. Core protein VII and pre-VII, and GST-tagged proteins were expressed and purified as previously described [16,17].

2.2. Immunoprecipitation assay

HeLa cells were infected with HAdV-5 at multiplicity of infection (MOI) of 10. At 24 h post infection (h.p.i.), cells ($1X10^7$) were collected and lysed on ice for 10 min in 1 ml of IP buffer (50 mM Tris-HCl pH 7.9, 1 mM PMSF, 0.1% Triton X-100, and 1 mg/ml bovine serum albumin (BSA)) containing 150 mM NaCl followed by extensive sonication. Cell extracts recovered as a supernatant fraction by centrifugation were mixed with anti-protein V (a generous

1 gift from Dr. W. C. Russel), anti-core protein VII [16], or anti-B23 (Zymed) antibodies, and
2 incubated at 4°C for 3 h. Then, Protein A Sepharose CL4B beads (10 μl of resin; Amersham
3 Pharmacia) were added and further incubated for 1 h with gentle agitation. Immunoprecipitated
4 proteins were analyzed by SDS-PAGE followed by western blotting.
5
6 *2.3. Electrophoretic mobility shift assay*
7 The 147 bp-long DNA fragment containing the *5S rRNA* gene was amplified by PCR.
8 DNA was first mixed with core proteins, and then increasing amounts of B23.1 were added, or
9 core proteins pre-incubated with increasing amounts of B23 were mixed with DNA. Both
10 reactions were carried out at 37°C for 15 min in a buffer containing 40 mM Tris-HCl pH 7.9, 100
11 mM NaCl, 0.1 mg/ml of BSA, and 10% glycerol and protein-DNA complexes were analyzed by
12 6% PAGE in 0.5XTBE.
13
14

3. Results and Discussion

3.1. Interaction of B23 with core proteins V and VII in Ad infected cells

To explore the function of B23 in the Ad life cycle, we first addressed whether B23 interacts with viral core proteins by immunoprecipitation experiments. HeLa cells were infected with HAdV-5, and cell lysates were prepared from cells at 24 h.p.i. Immunoprecipitation assays were carried out with antibodies against B23, core protein V, and core protein VII. Western blot analyses revealed that both core protein V and pre-VII were co-immunoprecipitated with B23 (Fig. 1A, lane 6). We also confirmed this interaction by immunoprecipitation assays using anti-core protein VII and V antibodies (Fig. 1B, lanes 1-6 and lanes 7-12, respectively) followed by western blotting with anti-B23 antibody. To further examine whether the interaction between B23 and basic core proteins was the consequence of artificial association between acidic and basic proteins, immunoprecipitation assays with antibodies against TAF-Iβ and B23 were carried out. TAF-I is a highly acidic protein that was identified as a stimulatory factor for the Ad DNA replication [18]. As shown in Figs. 1A and B, both core protein V and pre-VII were co-immunoprecipitated with B23, whereas only pre-VII was co-immunoprecipitated with TAF-I (Fig. 1C). The theoretical isoelectric point (pI) of TAF-I (4.12) is lower than that of B23.1 (4.64). In addition, we previously found that small acidic protein pp32 with theoretical pI of 3.99 is not associated with pre-VII in infected cell extracts [19]. Therefore, these indicate that the interaction between B23 and viral core proteins is not explained simply by non-specific charge-mediated interaction. Although our anti-VII antibody recognizes both core protein VII and pre-VII as shown in input extracts (Fig. 1A, lane 4), only pre-VII was found to be co-immunoprecipitated with B23. It was reported that immature virions termed sub-viral particles containing viral precursor proteins were assembled and form a distinct compartment in the nucleus before maturation of infectious virions [20]. In sub-viral particles, the viral precursor proteins including pre-VII are processed by a viral encoded protease [20]. Core protein VII

1 accumulated at the late stage of infection is present in the sub-viral particles, and thereby cellular
2 proteins were not accessible. In other words, cellular proteins associated with viral proteins
3 outside sub-viral particles should be dissociated prior to formation of sub-viral particles. Since
4 pre-VII is processed by Ad protease after deposition on the viral genome and during packaging
5 into sub-viral particles/virions, it is suggested that B23 plays a role in deposition of pre-VII to
6 DNA.

7 In order to test whether the interaction between B23 and core proteins in infected cells is
8 direct, GST-pull down assays were carried out using purified recombinant proteins (Figs. 1D-F).
9 The interaction between core protein V and GST proteins were detected by western blotting using
10 anti-core protein V antibody. GST-B23.1 and GST-B23.2, but not GST alone, pulled down core
11 protein V (Fig. 1E). Similarly, core protein VII and pre-VII were pulled-down with GST-B23.1
12 (Fig. 1F, lanes 5 and 6). Therefore, the interaction between B23 and viral core proteins is direct.

13

14 *3.2. B23 is co-fractionated with core protein V and pre-VII free of DNA*

15 During DNA replication in eukaryotic cells, newly replicated DNA is packaged into
16 chromatin with histones synthesized concomitantly with DNA replication. Chromatin assembly
17 of newly replicated DNA is mediated by a class of proteins termed histone chaperones [21]. We
18 showed that B23 functions as a histone chaperone [17]. To investigate a role of B23 in formation
19 of Ad DNA-core protein complexes, we examined whether B23 interacts with core proteins free
20 of Ad DNA in infected cells. At 24 h.p.i. when both core protein V and pre-VII are efficiently
21 synthesized and their assembly to newly replicated Ad DNA has started, cell extracts were
22 prepared and subjected to sucrose density gradient centrifugation (Fig. 2A). In mock-infected cell
23 extracts, B23 was broadly distributed with two peaks, one major peak in fractions 7-14 (peak I)
24 and the other minor peak in fractions 18-21 (peak II). Upon Ad infection, B23 in the peak I was
25 slightly shifted toward the bottom and the amount of B23 recovered in the peak II increased

significantly. The Ad DNA was examined by PCR with a primer set to amplify the *E1A* region [16]. In the peak II, core proteins and the viral genome were co-fractionated with B23. Since pre-VII is processed during maturation of viral DNA-core protein complexes, the peak II is likely to contain the premature and mature Ad core. Low but distinct amounts of core protein V and pre-VII were also found in the peak I in which the viral genome was not present (Fig. 2A, bottom panel). B23 in the peak I was co-immunoprecipitated with core protein V and pre-VII (Fig. 2B, lanes 4 and 8). These results suggest that B23 plays at least two distinct roles in viral chromatin assembly with core proteins either free of DNA or associated with the viral genome.

3.3. Biochemical characterization of B23 as a chaperone for viral core proteins

Sucrose density gradient assays demonstrated that B23 co-fractionated with viral core proteins free of DNA. This complex is assumed to be an intermediate during transfer of core proteins to DNA. This point was addressed by biochemical analyses using purified proteins. First, we tested the chaperone activity of B23 for core protein V (Figs. 3B and 3C). Two sets of experiments were performed. First, purified core protein V pre-incubated with DNA was mixed with increasing amounts of B23.1 (Fig. 3B). Second, purified core protein V pre-incubated with increasing amounts of B23.1 was mixed with DNA (Fig. 3C). DNA-protein complexes were analyzed by native PAGE. When mixed directly, DNA-core protein V complexes were distributed as diffuse bands with a high intensity at the top of the gel, suggesting that core protein V binds DNA randomly (Fig. 3B, lane 3). B23 shifted the bands toward the bottom of the gel and increased the amount of free DNA in a dose-dependent manner, indicating that B23 dissociates an excess amount of core protein V from DNA-core protein V complexes. Similarly, when core protein V was pre-incubated with B23, B23 restricted the access of core protein V to DNA and suppressed the formation of random DNA-core protein V complexes in a dose-dependent manner

1 (Fig. 3C). These results indicate that B23 associates with core protein V either complexed with
2 or free of DNA to decrease the amount and level of aggregation.
3 Next, we tested the ability of B23 as a chaperone for pre-VII (Figs. 3D and 3E). When
4 first mixed with DNA, pre-VII binds to DNA and forms a large aggregate that did not enter the
5 gel under the condition employed (Fig. 3D, lanes 2 and 7). However, B23 dissolved the
6 aggregates containing DNA and pre-VII, and free DNA increased in a B23 dose-dependent
7 manner. Interestingly, when DNA-pre-VII aggregates were incubated with high doses of B23, an
8 intermediate band appeared (white arrowhead). Since B23 alone did not bind to DNA (Figs. 3B
9 and 3C, lane 1), it is possible that some form of pre-VII-DNA complex was formed in the
10 presence of high doses of B23. When pre-VII was first pre-incubated with increasing amounts of
11 B23, association of pre-VII with DNA was restricted and the amount of free DNA increased (Fig.
12 3E). Furthermore, the intermediate band appeared, suggesting that a fraction of pre-VII incubated
13 with high doses of B23 was transferred to DNA. We also found that B23ΔC3 that lacks the
14 region essential for the association of B23 with pre-VII ([14] and data not shown) could not
15 dissolve the aggregated DNA-pre-VII complex (Fig. 3F). This indicates that the association
16 between B23 and pre-VII is important for regulation of the structure of the DNA-pre-VII complex.
17 To verify that B23 mediates deposition of pre-VII on DNA, [^{32}P]-labeled 147 bp DNA alone, and
18 pre-VII-B23 complexes incubated without or with [^{32}P]-labeled DNA were subjected to glycerol
19 density gradient (Fig. 4). Pre-VII pre-incubated with B23 in the absence of DNA sedimented
20 mainly in fraction 4 (Fig. 4A). When pre-VII-B23 complex was incubated with DNA, the peak of
21 pre-VII was slightly shifted toward the bottom and a minor population of pre-VII was recovered
22 in the pellet fraction. The intermediate shifted band was detected in fractions 4-6 on native
23 PAGE. To examine whether this band shift is due to the complex formation with pre-VII, each
24 glycerol density gradient fraction was subjected to immunoprecipitation without or with anti-VII
25 antibody. As clearly shown in Fig. 4B, DNA was co-precipitated with pre-VII in fractions 2-6.
26 Thus, we concluded that B23 can mediate the formation of the nucleoprotein complex containing

pre-VII. These biochemical analyses altogether support an idea that B23 functions as a chaperone during viral chromatin assembly in infected cells.

In this manuscript, we described a function of B23 in viral chromatin assembly at the late stage of infection. In order to understand how B23 mediates the viral chromatin assembly, more detailed studies are needed. However, based on our results we would propose that B23 is involved in viral chromatin assembly by two distinct mechanisms. First, B23 may bind to newly synthesized core proteins and transfers them to DNA to assemble viral chromatin. This is supported by the results that B23 is associated with core proteins in infected cell extracts (Figs. 1 and 2) and B23 mediates transfer of core proteins to DNA (Figs. 3 and 4). The other is that newly synthesized core proteins may bind with the viral genome first and B23 may regulate the amount of core proteins associated with viral genome (Figs. 3B and D). To fully assemble the Ad core, it is important to clarify how core protein V and pre-VII are deposited on one DNA molecule. In addition, viral core protein X/mu is also known to be associated with viral genome. Thus, we have to take into consideration of this small core protein. Recently, we have shown that TAF-I plays an important role in the early stages of adenovirus infection [19]. Since B23 was identified as an activity similar to that of TAF-I, it is also possible that B23 plays an important role in the early stage of infection. The function of B23 at the early stage of Ad infection is under investigation.

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32

1 Figure legends
2

3 Fig. 1. Interaction between B23 and core proteins in infected cells. A, B: Immunoprecipitation
4 from Ad-infected cell extracts. Cell lysates prepared from mock- or Ad-infected HeLa cells were
5 subjected to immunoprecipitation without or with anti-B23 antibody (A) or anti-core protein V
6 and anti-core protein VII antibodies (B). B23 and core proteins VII and V were detected by
7 western blotting. Positions of the proteins were indicated at the right side of the panel. Asteisks
8 indicate the immunoglobin heavy chain. C: Immunoprecipitation with anti-TAF-I and anti-B23
9 antibodies. Cell lysates prepared from mock (lane 1) and Ad-infected cells (lanes 2-4) were
10 subjected to immunoprecipitation without (lane 3) or with anti-TAF-Iβ [19] (lanes 1 and 2) or
11 anti-B23 (lane 4) antibody. Proteins were separated on 10% SDS-PAGE followed by western
12 blotting with anti-core protein V or VII antibodies (top and bottom panels, respectively). D:
13 Purified recombinant proteins. Purified GST, GST-B23.1, GST-B23.2, and His-core protein V
14 (lanes 1-4, respectively) were separated on a 10% SDS-PAGE and visualized by CBB staining.
15 Lane M indicates molecular weight markers. E: GST-pull down assays with core protein V. GST,
16 GST-B23.1, and GST-B23.2 (lanes 2-4, respectively) (1 μg each) were incubated with His-core
17 protein V (1 μg) in IP buffer containing 200 mM NaCl and incubated at 4°C for 1 h. Then 20 μl
18 of glutathione Sepharose CL4B beads (Amersham Pharmacia) were added and further incubated
19 for 1 h. Proteins bound to glutathione-Sepharose beads were separated on a 12.5% SDS-PAGE
20 and analyzed by western blotting with anti-core protein V antibody. Purified core protein V (0.1
21 μg, lane 1) was also shown. F: GST-pull down assays with core protein VII and pre-VII.
22 Purified pre-VII (lanes 1, 3, and 5) and core protein VII (lanes 2, 4, and 6) (1 μg each) were
23 mixed with either GST or GST-B23.1 (1 μg each) as in Fig. 1E. Proteins bound to Glutathione-
24 Sepharose beads were separated on a 12.5% SDS-PAGE and visualized by CBB staining. Lane
25 M indicates molecular weight markers. Positions of GST, GST-B23.1, pre-VII and core protein
26 VII were indicated at the left side of the panel.

1

2 Fig. 2. Two forms of B23-core protein complexes. A: Sucrose density gradient. HeLa cells
3 were infected with HAdV-5 at MOI of 10. At 24 h.p.i., mock- and Ad-infected HeLa cells were
4 collected, suspended in hypotonic buffer (10 mM Hepes NaOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM
5 KCl, and 0.5 mM DTT), and incubated on ice for 10 min. Then, cells were disrupted by
6 homogenization, and soluble cell extracts were recovered by centrifugation. Extracts were
7 dialyzed against 20 mM Tris HCl pH 7.9, 100 mM NaCl, 10% glycerol, 0.5 mM DTT, and 0.5
8 mM PMSF and loaded on 2.2 ml of 10-60% sucrose density gradient. Samples were centrifuged
9 in TLS55 rotor (Beckman) at 50,000 rpm for 6.5 h. Ten microliter of each fraction (100 μl)
10 recovered from the top was separated on a 12.5% SDS-PAGE followed by western blotting using
11 antibodies against B23, core proteins V, and VII. The viral DNA was purified from each fraction
12 and detected by PCR (bottom panel). B: Interaction of B23 with core protein V and pre-VII free
13 of the Ad DNA. Sucrose density gradient fractions 9-11 from mock- or Ad-infected extracts were
14 subjected to immunoprecipitation with anti-core protein V (lanes 3 and 4) and anti-VII (lanes 7
15 and 8) antibodies. Immunoprecipitated proteins were separated on a 10% SDS-PAGE followed
16 by western blotting with anti-B23 antibody. The position of B23 is indicated by arrowheads.
17 Asterisks indicate the immunoglobulin heavy chain.
18

19 Fig. 3. Function of B23 as a chaperone for viral core proteins. A: Purified recombinant proteins.
20 Purified pre-VII and His-B23.1 (lanes 1 and 2, respectively) were separated on a 12.5% SDS-
21 PAGE and visualized by staining with CBB. B, C, D, E, F: Roles of B23 in core protein-DNA
22 complex formation. Nucleoprotein complexes were resolved on a 6% PAGE in 0.5 X TBE and,
23 DNA was visualized with SYBER-Gold staining. (B) B23 dissociates excess amounts of core
24 protein V from the DNA-core protein V complex. Purified core protein V (50 ng) pre-incubated
25 with 147 bp-long DNA (50 ng) was mixed without (lane 3) or with increasing amounts of B23.1

(50, 100, 200, and 400 ng for lanes 4-7, respectively) and further incubated. DNA either with B23.1 (200 ng) or alone (lanes 1 and 2, respectively) was also loaded. (C) B23 suppresses the formation of random DNA-core protein V complex. Purified core protein V pre-incubated without or with increasing amounts of B23.1 was mixed with DNA as in panel B. (D) B23 dissociates pre-VII-DNA aggregates. Purified pre-VII (50 ng for lanes 2-6 and 100 ng for lanes 7-11) pre-incubated with DNA (50 ng) was mixed without (lanes 2 and 7) or with increasing amounts of B23.1 (50, 100, 200, and 400 ng for lanes 3-6 and 8-11, respectively) and further incubated. Free DNA (lane 1) was also shown. (E) B23 associates pre-VII to DNA. Pre-VII pre-incubated without or with increasing amounts of B23.1 was mixed with DNA as in panel D. (F) B23ΔC cannot dissolve the aggregated pre-VII-DNA complex. DNA (50 ng) and pre-VII (100 ng) were pre-incubated, and increasing amounts of B23.1 (0, 50, 100, 200 ng for lanes 2-5) or B23ΔC (0, 50, 100, 200 ng for lanes 7-10) were added and further incubated. Free DNA (lane 1 and 6) was also shown.

Fig. 4. Transfer of pre-VII to DNA by B23. A: Glycerol density gradient assay. pre-VII (2 μg)-B23 (25 μg) complexes in the absence or presence of [^{32}P]-labeled 147 bp DNA (2 μg) were subjected to centrifugation at 50,000 rpm for 4 h in TLS 55 rotor on a 10-40% glycerol gradient, and fractions were recovered from the top. After fractionation, remaining proteins in the pellet of the tube were suspended in 200 μl of buffer and recovered as a pellet fraction (P). Proteins in each fraction were analyzed by western blotting, and [^{32}P]-labeled DNA was analyzed by a 6% native PAGE. B: Immunoprecipitation of glycerol density gradient fractions. Each fraction from the pre-VII-B23-DNA complex and [^{32}P]-labeled DNA alone was counted with scintillator (top panel), and each fraction was subjected to immunoprecipitation without or with anti-core protein VII antibody. The radioactivity of immunoprecipitated DNA was analyzed with scintillator (bottom panel).

Fig. 1

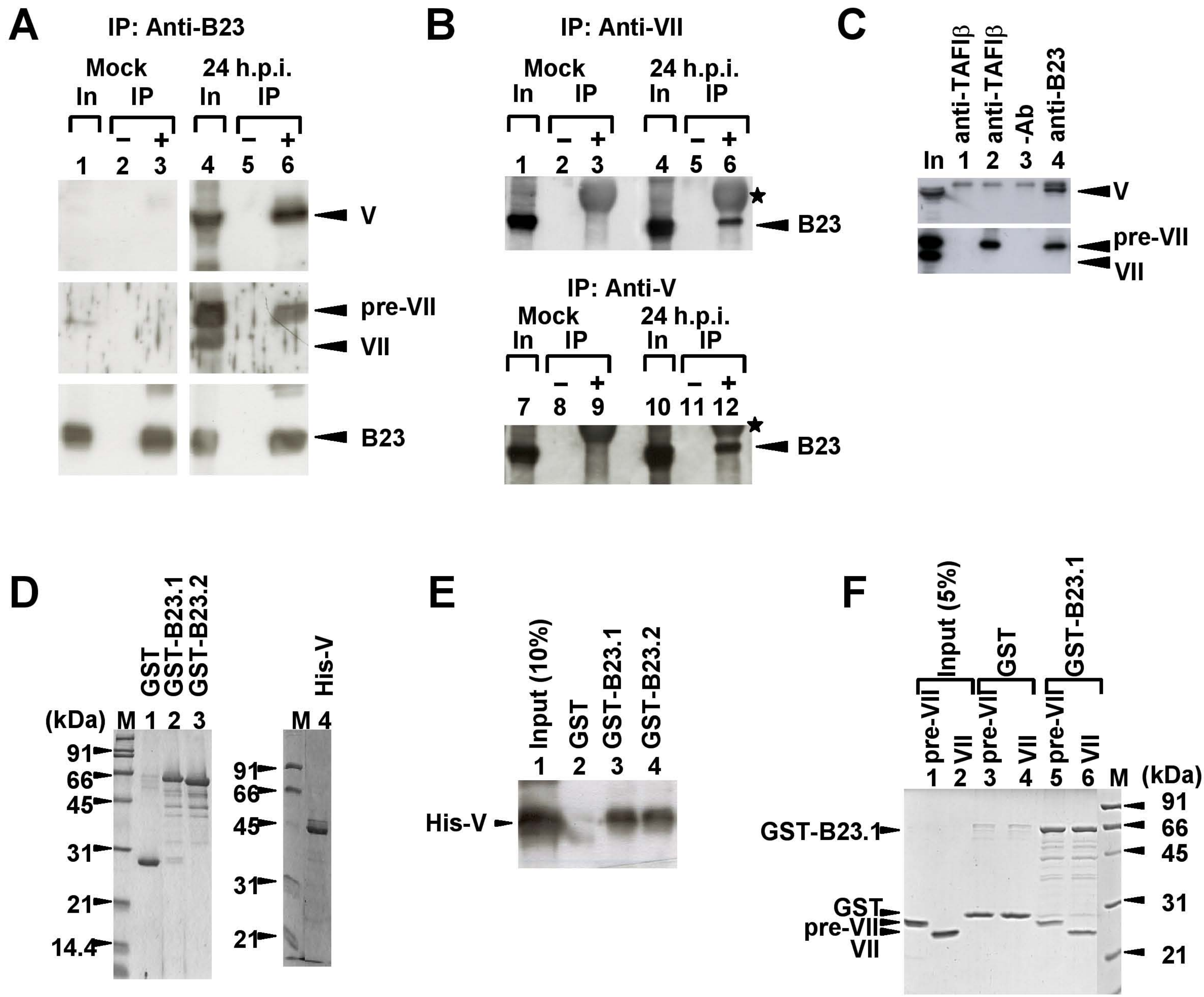


Fig. 2

A

Top Bottom

In 1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17 18 19 20 21 22

Mock — B23

Infected — B23, V, pre-VII, VII, Viral DNA

B

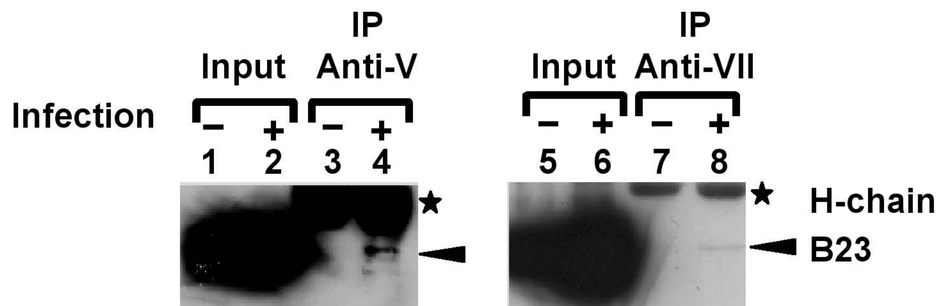


Fig. 3

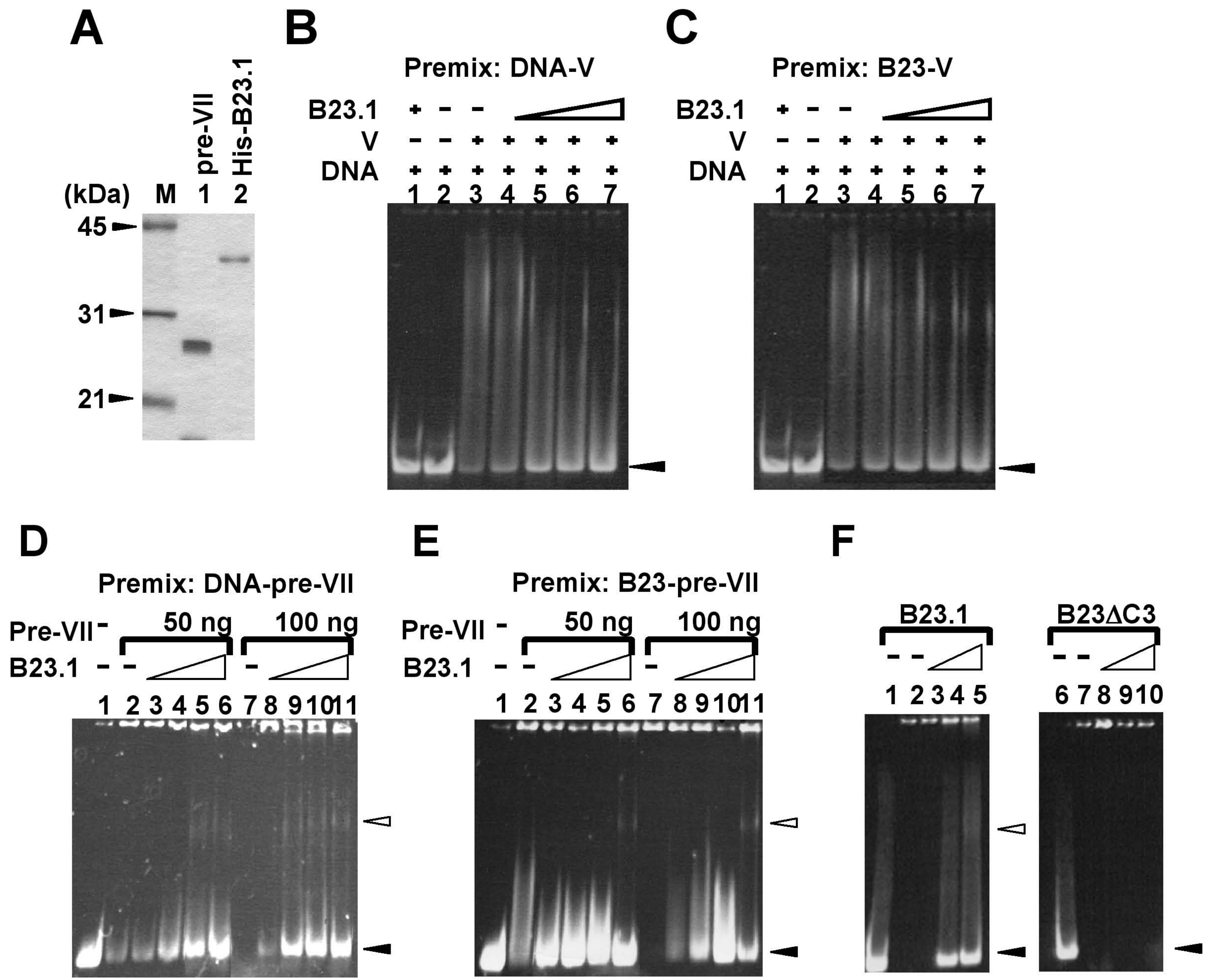
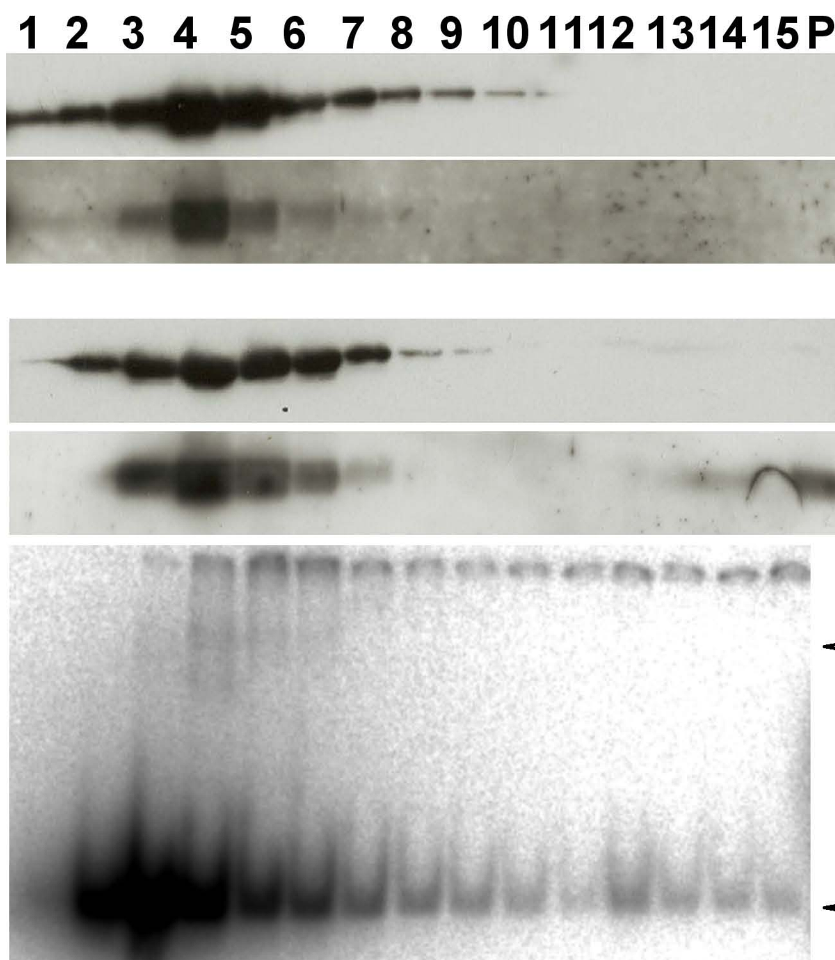


Fig. 4

A



B