B23/nucleophosmin is involved in regulation of adenovirus chromatin structure at late infection stages, but not in virus replication and transcription

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INTRODUCTION

Adenovirus is an icosahedral particle with a linear dsDNA of approximately 36 000 bp. The linear DNA is linked covalently to virus-encoded terminal proteins and condensed with the viral basic proteins Mu, VII and V, thus forming a chromatin-like structure termed adenoviral core/chromatin (Anderson et al., 1989; Black & Center, 1979; Chatterjee et al., 1985). Protein VII, a 19 kDa basic protein, is the major component of the adenoviral core and is most tightly associated with the genome (Sung et al., 1983). Protein V associates loosely with the viral DNA and forms an outer shell around the core to link it to 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 the viral DNA–VII hexamer complex is bridged by core protein V (Déry et al., 1985; Sung et al., 1977).

Infecting adenovirus particles are disassembled in the cytoplasm in a stepwise manner after penetration through endocytosis, and the viral core enters 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 become dissociated from the 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 has been 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 remodelled after entry into the host nucleus (Chen et al., 2007; Matsumoto et al., 1993, 1995; Spector, 2007). Histones may associate with the incoming viral DNA (Sergeant et al., 1979; Tate & Philipson, 1979). Recently, it has been shown that, as well as protein VII, cellular histones are also functional components of viral chromatin in the early phases of infection (Komatsu et al., 2011). During the late stages of infection,
the precursor of core protein VII (pre-VII) and core protein V are synthesized at high levels concomitant 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 (Déry et al., 1985). However, mature adenovirus 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 remodelled the adenoviral core structure and stimulated replication and transcription from the core (Matsumoto et al., 1993, 1995; Okuwaki et al., 2001a). Recently, we have shown that TAF-I remodels the core structure by forming a ternary complex with adenoviral DNA–core protein VII complexes and plays an important role in the early stages of the adenovirus infection cycle (Gyurcsik et al., 2006; Haruki et al., 2003, 2006; Komatsu et al., 2011). TAF-II is identical to nucleosome 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., 2001b, 2005) 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, whilst the C-terminal region unique for B23.1 is essential for its RNA-binding activity. Recently, we have shown that B23 interacts with adenoviral core proteins V, VII and pre-VII, and may have a role as a chaperone in the assembly of core proteins into the viral core (Samad et al., 2007). However, an in vivo role(s) of B23 in the adenovirus life cycle has not yet been clarified. Here, we developed a decoy molecule for the interaction between B23 and core protein V based on analysis of the interaction domains between B23 and core protein V. First, we determined the domain of core protein V required for its interaction with B23. Core protein V contains lysine- and arginine-rich basic clusters in its N- and C-terminal regions. We postulated that core protein V would 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. 1(a). GFP- and Flag-tagged core protein V mutants were co-expressed with haemagglutinin (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, bound similarly to HA–B23.1 (Fig. 1b, lanes 11 and 12). However, 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 (Fig. 1b, lanes 13 and 14). In contrast, the N- and C-terminal fragments, in the form of mutants V(1–78) and V(314–369), respectively, efficiently co-precipitated HA–B23.1 (Fig. 1b, lanes 15 and 16). These results indicated that both N- and C-terminal regions are involved in the interaction between B23 and core protein V, and that these fragments would be good candidates for decoy molecules for the interaction of core protein V with B23.

### Inhibition of infectious virus production by B23 decoy molecule

We hypothesized that overexpression of these domains would interfere with the function of B23 in adenovirus proliferation if B23 is involved in the virus life cycle. To test this, HeLa cells were transfected with GFP-empty vector or with vectors for the expression of GFP–V, GFP–V(1–78), GFP–V(79–313) and GFP–V(314–369), and superinfected with human adenovirus type 5 (HAdV5) at 20 h post-transfection. At 24 h post-infection (p.i.), progeny virus particles were collected and the infectivity titre was determined as described in Methods [Figs 1c and S1 (available in JGV Online)]. The results demonstrated that over-expression of GFP–V, GFP–V(1–78) and GFP–V(314–369), but not of GFP alone or GFP–V(79–313), inhibited the production of infectious virus particles. These results suggested that the mutant proteins that tightly associated with B23 inhibited the infectious virus production. It was
further demonstrated that co-expression of exogenous B23 with GFP–V(1–78) (Fig. 1d) rescued the negative effect of GFP–V(1–78). These results supported the idea that V(1–78) functions as a type of decoy for the interaction between B23 and core protein V, and that impairment of this interaction decreases the progeny virus production level.

B23 knockdown inhibits the production of infectious viral particles

To further demonstrate that B23 is involved in adenovirus 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 adenovirus replication (Hindley et al., 2007), we focused on B23.1, as it is concentrated in the nucleolus, where core protein V is located at the late stage of infection (Matthews, 2001), whilst B23.2 is distributed throughout the nucleus. In addition, depletion of B23.1 alone was shown to efficiently decrease 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 that of β-actin, whereas control siRNA had no effect (Fig. 2a). The expression levels of another host factor for adenovirus remodelling, TAF-I, and other nucleolar proteins such as

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**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 constructs. GFP–Flag tag was fused at the N terminus of core protein V and its mutant proteins. The basic clusters of core protein V are indicated by filled boxes. (b) Immunoprecipitation of Flag-tagged core protein V. 293T cells were transiently co-infected with HA-B23.1 and either GFP–Flag–protein 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 by SDS-PAGE (12.5% acrylamide) and detected by Western blotting with anti-HA and anti-Flag antibodies (top and bottom panels, respectively). The positions of GFP–Flag-tagged protein V and its mutants are indicated by arrowheads on the left of each lane. (c) Inhibition of virion production by a decoy molecule. HeLa cells were transfected with GFP-empty vector or a vector expressing GFP–Flag-tagged V(1–78) mutant. At 20 h post-transfection, cells were infected with HAdV5. At 24 h p.i., virus in the culture fluid was collected and virus titre was determined. In the lower panel, expression of exogenous proteins as well as β-actin was confirmed by Western blotting. (d) Rescue experiments. Cells were transfected with 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 h post-transfection, the cells were superinfected with HAdV5 and virus production was examined as described in (c). Exogenously expressed proteins were detected by Western blotting, shown in the lower panel.
nucleolin and fibrillarin were found to be unchanged following B23 knockdown (Fig. 2b). To examine the effect of B23 knockdown on adenovirus proliferation, control and B23.1 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, DNA-binding protein (DBP) was concentrated in nuclear foci, whilst core protein VII was distributed throughout the nuclei at 24 h p.i. As reported previously (Hindley et al., 2007; Matthews, 2001), the nuclear localization of B23 was slightly suppressed following adenovirus 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 h p.i., the supernatant fraction containing progeny virus particles was collected, cleared by low-speed centrifugation and examined to determine the infectious titre (Fig. 2d). The production of infectious progeny virus particles from B23 knockdown cells was decreased to approximately 50–60 % of that from control siRNA-treated cells (Fig. 2d, lanes 1 and 2). Even with the decreased B23.1 level, adenovirus production increased until 48 h p.i., although the amount of infectious virus produced from 24 to 36 h p.i. was lower than that in the control siRNA-treated cells (Figs 2c and S2). These results suggested that B23.1 is not essential but plays a crucial role in adenovirus production, and/or that other cellular factor(s) could also be involved (see Discussion). Next, we investigated whether the effect of B23 knockdown on adenovirus production could be rescued by B23 overexpression. At 36 h after the introduction of control or B23 siRNA, cells were transfected with empty vector or with vector encoding HA–B23.1. Cells were then infected with HAdV5 at 24 h after transfection of plasmid DNA and the production of progeny virus particles was examined. The expression levels of exogenous HA–B23.1 are shown in Fig. 2(e). Overexpression of B23.1 in control cells slightly inhibited infectious progeny virus production (Fig. 2d), although this result was not statistically significant. Importantly, exogenous expression of B23.1 counteracted the negative effect of B23.1 siRNA-mediated knockdown on progeny virus production. These results support the idea that B23.1 plays an important role in the production of infectious virus particles.

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

Given that B23 knockdown decreased the production of infectious virus particles, it was expected that this inhibition might be due to interference with viral DNA replication. To test this notion, control or B23 siRNA-treated HeLa cells were infected with HAdV5 and the amplification of viral DNA at 12, 18 and 24 h p.i. was examined by quantitative PCR (qPCR) using a primer set specific for adenviral DNA (Fig. 3a). Because the amount of viral DNA increased as a function of incubation period after infection and the amplification of DNA was strongly inhibited by hydroxyurea, an inhibitor of DNA synthesis, it was confirmed that the PCR products detected under the conditions employed here corresponded to the amount of viral DNA. Surprisingly, no significant decrease in the amount of viral DNA was observed following B23 knockdown (Fig. 3a). We also examined the effect of B23 knockdown on viral late gene expression. The expression level of late genes was examined by Western blotting with anti-pVII and anti-V antibodies (Fig. 3b) and by RT-PCR with primer sets for mRNAs of the major late promoter (MLP) and pVII (Fig. 3c, d). Consistent with the fact that late gene transcription depends on viral DNA replication, the expression level of late genes was strongly inhibited by the presence of hydroxyurea. We did not find any significant decrease in mRNA and core protein expression levels following B23 knockdown (Fig. 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. As adenovirus genome replication depends completely on viral early gene products, we could exclude the possibility that B23 is involved in early gene transcription.

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

Our biochemical data suggested that the adenoviral core proteins form aggregates with viral DNA when mixed directly, and that B23 dissociates the aggregation between the 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 knockdown affects the virus genome chromatin structure in infected cells. To test this possibility, we examined whether B23 was associated with the virus genome in infected cells. HeLa cells were infected with HAdV5, and at 20 h p.i. cells were cross-linked with formaldehyde and then sonicated to release chromatin. The mean 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 and VII or B23. We found that B23 associated with the virus genome (the VA gene region), as did the core proteins V and VII (Fig. 4a). We examined the association of B23 with the virus genome using primer sets, as shown in Fig. 4(b, lower panel). 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 m.o.i. of 10. At 20 h p.i., cells were subjected to ChIP assays, as described above. Five different primer sets, as shown in Fig. 4(b), were used to examine the amounts of core proteins and histones on the virus genome. In B23 knockdown cells, association of the core proteins V and VII with viral DNA was found to be increased in all regions tested (Fig. 4c, d). We also found that the association of histone H3 along the virus genome was increased (Fig. 4e).
We also examined whether the amounts of core proteins and histone H3 on the virus genome increased by B23.1 knockdown 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 HAdV5. At
24 h p.i., ChIP assays were carried out as shown in Fig. 4 using primer sets specific for the VA gene region (Fig. 5). Western blotting analyses demonstrated that the amount of B23.1 was decreased efficiently by siRNA treatment and was recovered by transient expression of exogenous HA–B23.1 (Fig. 5a, lanes 5 and 6). Consistent with the results in Fig. 4, the amounts of core proteins V and VII and histone H3 on the virus genome were increased by B23.1 knockdown (Fig. 5b–d). However, they were decreased following overexpression of HA–B23.1. Interestingly, even in control siRNA-treated cells, HA–B23.1 overexpression decreased the association levels 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 studied the in vivo function of B23 in the adenovirus life cycle. Based on previous reports, it was expected that B23 might be involved in adenoviral 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) or the level of transcription or translation of core proteins (Fig. 3b–d) following B23.1 knockdown. Therefore, it is possible that B23.1 is not involved in adenoviral DNA replication in infected cells under the conditions used here or that loss of B23.1 may be compensated alternatively. In this sense, it should be noted that, as well as B23, other histone chaperones have also been identified as factors for adenoviral DNA replication (Kawase et al.,...
In addition, we could not exclude the possibility that B23.2 that remains in B23.1 knockdown cells plays a compensatory role in adenoviral DNA replication. Nevertheless, the data presented here demonstrated that B23.1 knockdown did not significantly affect DNA replication and transcription.

We showed that the decrease in B23 reduced the production of progeny virions and increased the association level.
levels of B23 and (lanes 5 and 6) and then infected with HAdV5. The expression of endogenous and exogenous B23.1. HeLa cells were treated with control (C) or B23.1 (Si) siRNA without (lanes 1 and 2) or with transfection of pCHA (lanes 3 and 4) or pCHA-B23.1 (lanes 5 and 6) and then infected with HAdV5. The expression levels of B23 and β-actin were examined by Western blotting. (b–d) ChIP assays. HeLa cells prepared as described in (a) were subjected to ChIP assays with antibodies against core proteins V (b) or VII (c) or histone H3 (d). Immunoprecipitated DNA was examined quantitatively by qPCR using primer sets specific for the adenovirus VA gene region. The amount of immunoprecipitated DNA was analysed quantitatively and compared with the DNA extracted from input extracts. PCRs were carried out in triplicate and results are shown as means ± SD.

Fig. 5. Overexpression of exogenous B23.1 counteracts the effect of B23.1 knockdown on viral chromatin structure. (a) Expression levels of endogenous and exogenous B23.1. HeLa cells were treated with control (C) or B23.1 (Si) siRNA without (lanes 1 and 2) or with transfection of pCHA (lanes 3 and 4) or pCHA-B23.1 (lanes 5 and 6) and then infected with HAdV5. The expression levels of B23 and β-actin were examined by Western blotting. (b–d) ChIP assays. HeLa cells prepared as described in (a) were subjected to ChIP assays with antibodies against core proteins V (b) or VII (c) or histone H3 (d). Immunoprecipitated DNA was examined quantitatively by qPCR using primer sets specific for the adenovirus VA gene region. The amount of immunoprecipitated DNA was analysed quantitatively and compared with the DNA extracted from input extracts. PCRs were carried out in triplicate and results are shown as means ± SD.

of viral core proteins and cellular histones on the progeny virus genome DNA. Based on these observations, we propose that: (i) B23 is involved in the adenovirus infection cycle at a step after late gene expression, and (ii) proper virus chromatin assembly is required for adenovirus virion maturation. The precise mechanism of how B23 regulates viral chromatin and is involved in the final maturation step of infectious virus particles remains unclear. It is also possible that, in addition to B23, another cellular factor(s) is involved in these processes. TAF-I is a candidate for such an additional factor, as we have reported that TAF-I is also associated with pre-VII in the late phases of infection (Gyurcsik et al., 2006). It is thought that the viral DNA associated with appropriate amounts of basic proteins is important for encapsidation. In fact, the adenovirus genome DNA is condensed into a core structure only by viral basic proteins within virions, although viral DNA is associated with histones throughout the infection cycle (Déry 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 may 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 assumptions (Déry 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% FBS at 37°C. 293T cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (Nissui) containing 10% FBS. The HAdV5 used in this study was amplified and purified as described previously (Haruki et al., 2006).

Plasmid construction and transfection. The construction of plasmids for a series of protein V mutants is described in the Supplementary Methods. pCHA-B23.1 was prepared as described by 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 mAb for B23 that recognizes endogenous B23.1 was purchased from Invitrogen. Polyclonal antibody recognizing both B23.1 and B23.2 was generated in rabbits using B23MC2 expressed in Escherichia coli (Okuwaki et al., 2001a) as an antigen. Rabbit anti-core protein V and
mouse anti-DBP antibodies were obtained from Professor W. C. Russell (University of St Andrews, Fife, UK) as a generous donation. The rat polyclonal anti-pre-VII (Haruki et al., 2003), mouse mAb against Flag tag, rabbit mAb against HA, rabbit polyclonal antibody against histone H3 and mouse mAb against β-actin have been described elsewhere (Murano et al., 2008).

**Immunoprecipitation assays.** 293T cells, transiently transfected with plasmids where indicated, were lysed in 1 ml immunoprecipitation buffer [50 mM Tris/HCl (pH 7.9), 1 mM PMSF, 0.1% Triton X-100, 1 mg BSA ml⁻¹] 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 h. Protein A-Sepharose beads (10 μl resin; GE Healthcare) were added and the mixture was further incubated for 1 h with gentle agitation. The beads were washed three times with 0.5 ml immunoprecipitation buffer. Immunoprecipitated proteins were eluted by the addition of SDS sample buffer, and boiled, separated by SDS-PAGE (12.5% acrylamide) and transferred to PVDF membrane (Millipore). The membrane was subjected to Western blotting analysis using anti-HA antibody.

**Decoy assays.** HeLa cells were transfected with GFP-empty vector or with vector for the expression of GFP–V(1–78). At 24 h post-transfection, cells were superinfected with HAdV5 at an m.o.i. of 10. At 24 h p.i., culture supernatant (5 ml) was added with PBS, fixed with 3% paraformaldehyde for 10 min at room temperature and then treated with 0.5% NP-40 in PBS for 5 min at room temperature. After blocking with 5% non-fat milk in TBS with 0.1% Tween 20, samples were subjected to immunofluorescence analyses using the antibodies described above. Proteins were visualized with secondary antibodies (AlexaFluor 488-conjugated anti-rabbit IgG, AlexaFluor 568-conjugated anti-mouse IgG and AlexaFluor 568-conjugated anti-rat IgG; Invitrogen). DNA was visualized by staining with TO-PRO-3 iodide (Invitrogen). Labelled cells were observed by confocal laser-scanning microscopy (LSM5 Exciter; Carl Zeiss) using argon laser (488 nm) and HeNe laser (546 and 633 mm) lines.

**qPCR.** Control and B23 siRNA-treated HeLa cells were infected with HAdV5 at an m.o.i. of 10. At 12, 18 and 24 h p.i., cells (1 × 10⁶) were collected and suspended in lysis buffer [20 mM Tris/HCl (pH 7.9), 100 mM NaCl, 5 mM EDTA, 0.5% SDS] and total DNA was purified by proteinase K treatment overnight at 50 °C, followed by phenol/ chloroform extraction and ethanol precipitation. The amount of DNA was then examined by qPCR with a primer set specific for the adenovirus VA gene region (see below). Total RNA was purified from infected cells (1 × 10⁶) using an 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 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 a Thermal Cycler Dice Real-time System (Takara) was performed using synthesized cDNA as template with primer sets specific for the mRNAs from the MLP (5’-ACCTCCTCTCGGATGCTGT-3’ and 5’-GTGACTGTGTAGACGCGTTCT-3’), and β-actin (5’-ATGGTGCTAGAAGATTCTCATGT-3’ and 5’-GGTCATCTTCTGCGGTT-3’) genes.

**ChIP assays.** ChIP assays were carried out as described by the manufacturer (ChIP Assay Kit; Millipore) with antibodies against core proteins V and pVII, B23 and histone H3. The amount of immunoprecipitated DNA was determined by qPCR as described above. The reaction conditions have been described previously (Komatsu et al., 2011). The following primer sets were used: 5’-GGGCTAAAGTGTCGTGGTATTAC-3’ and 5’-AAAAAGTGCTAGAACTGGA-3’ for the E1a promoter region, 5’-GGGTCCTCTCGTATAGAAGA-3’ and 5’-GGCCACCCGCTTATAGCC-3’ for the MLP region, 5’-GCTGAGGCGACAAAACCAATA-3’ and 5’-TATCTTGGCGGG-3’ for the E4 ORF region, and 5’-CCTCAACGCTGCTGGAAAA-3’ and 5’-ATGGTGCTAGAAGATTCTCATGT-3’. Protein A–Sepharose beads (10 μl resin; GE Healthcare) were added and the mixture was further incubated for 1 h with gentle agitation. The beads were washed three times with 0.5 ml immunoprecipitation buffer. Immunoprecipitated proteins were eluted by the addition of SDS sample buffer, and boiled, separated by SDS-PAGE (12.5% acrylamide) and transferred to PVDF membrane (Millipore). The membrane was subjected to Western blotting analysis using anti-HA antibody.

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