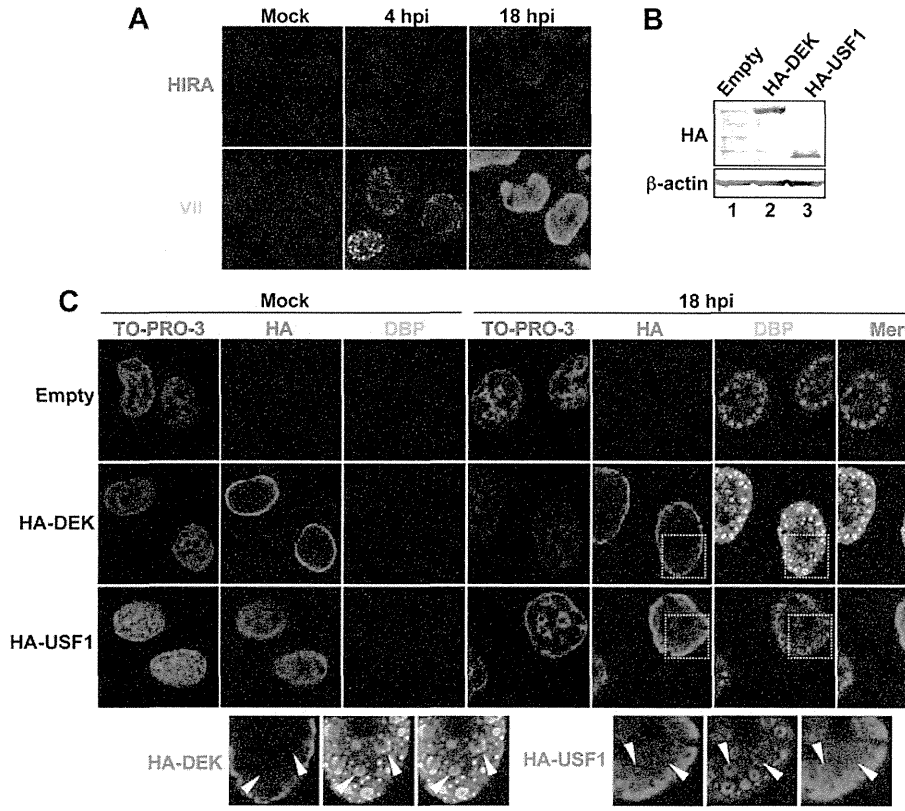

FIG 5 Localization of nuclear proteins in late phases of infection. (A) IF analyses using anti-HIRA antibody. HeLa cells were mock infected or infected with HAdV5 at an MOI of 250 (for 4 hpi) or 50 (for 18 hpi) and subjected to IF analyses using anti-protein VII and anti-HIRA antibodies. (B) Western blot analyses. Lysates were prepared from HeLa cells transfected with the pCHA-puro empty vector (lane 1), pCHA-DEK (lane 2), or pCHA-puro-USF1 (lane 3) at 24 hpt and were subjected to 10% SDS-PAGE, followed by Western blot analyses using anti-HA (top) and anti-β-actin (bottom) antibodies. (C) Localization of HA-DEK and HA-USF1. HeLa cells were transfected with the pCHA-puro empty vector, pCHA-DEK, or pCHA-puro-USF1. At 24 hpt, cells were mock infected or infected with HAdV5 at an MOI of 50, and at 18 hpi, the cells were subjected to IF assays using anti-HA and anti-DBP antibodies.

cated that histones are localized reciprocally to VDRF (and CAF-1/PCNA) in late phases of infection.

To gain more insights into the accessibility of other nuclear proteins to VDRF, we again performed IF analyses (Fig. 5). First, IF analyses were carried out by using antibody against HIRA, an H3.3-specific histone chaperone, and it was observed that the localization of HIRA was not drastically changed in both the early and late phases of infection (Fig. 5A). We also performed IF analyses using cells transiently transfected with expression vectors for HA-tagged DEK and USF1 (Fig. 5B and C). DEK is a cellular chromatin protein with potential histone chaperone activity (37), and USF1 is an E-box binding transcription factor and was reported previously to bind to and regulate transcription from the MLP region (46). HeLa cells were transfected with expression vectors, and at 24 hpt, the cells were subjected to Western blot analyses (Fig. 5B) or infected at an MOI of 50. At 18 hpi, the localization of DBP, HA-DEK, and HA-USF1 was visualized by IF analyses using anti-DBP and anti-HA antibodies (Fig. 5C). In mock-infected cells, both HA-tagged proteins showed a nuclear localization, and in the case of HA-DEK, a strong signal was observed at the nuclear periphery. At 18 hpi, HA-DEK appeared to be excluded from VDRF (Fig. 5C). However, in contrast to HA-DEK, we observed that HA-USF1 could be localized inside VDRF (Fig. 5C). Taken together, our IF analyses suggest that VDRF may allow the selective access of cellular nuclear proteins, and at least one of the transcription factors, USF1, is able to access VDRF.

Oligomerization of DBP is critical for histone exclusion from VDRF. To investigate the mechanism of histone exclusion from VDRF, we hypothesized that DBP may play a role, since an abundant amount of DBP was associated with Ad DNA in VDRF. The crystal structure of DBP revealed that this protein has a 17-aa extension at its C terminus (Fig. 6A), and this C-terminal "arm" hooks onto the next DBP molecule, resulting in the oligomerization of DBP (47). It was also reported previously that the oligomerization of DBP mediated by the C-terminal arm enables the ATP-independent unwinding of dsDNA, and thus, full-length DBP but not the deletion mutant that lacks the C-terminal arm (DBPΔC) could support viral DNA replication *in vitro* (9). Therefore, to examine a role of DBP and its oligomerization in histone localization, HeLa cells expressing histone H3.3-EGFP were transfected with the expression vectors for HA-tagged full-length DBP or DBPΔC, and at 36 hpt, the cells were subjected to Western blotting and IF assays using anti-HA and anti-DBP antibodies (Fig. 6B and C). The expression levels of both DBP proteins were almost the same, as indicated by Western blotting (Fig. 6B). By IF analyses, we observed that full-length DBP forms foci like VDRF

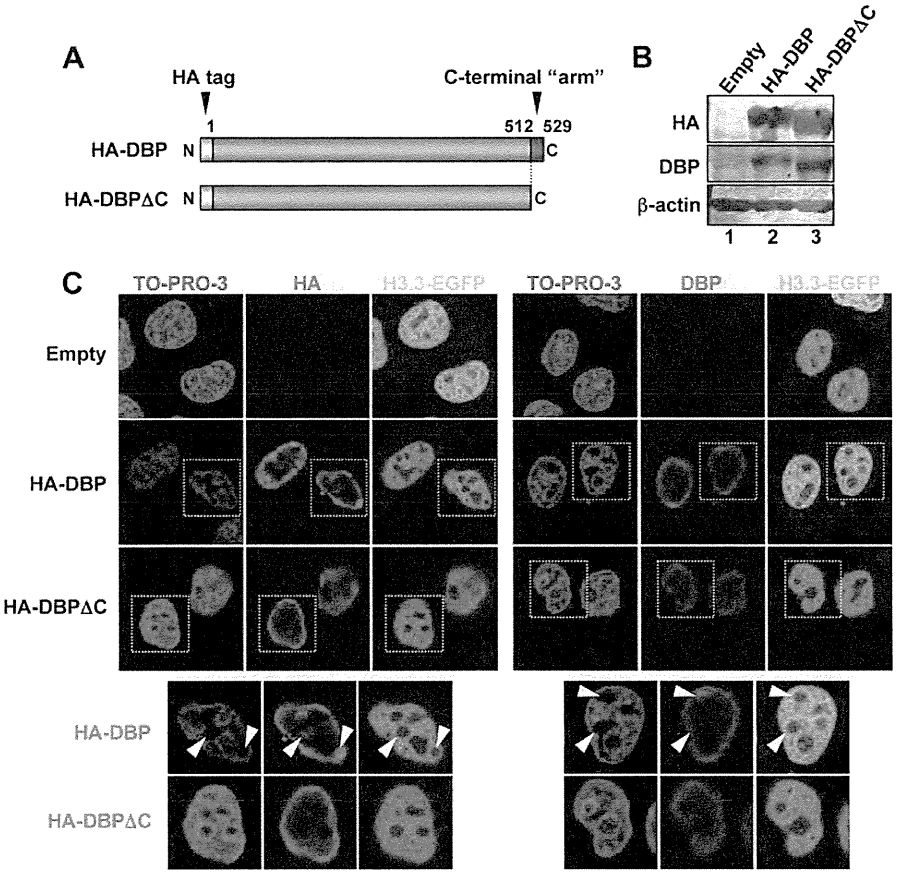

FIG 6 Role of DBP oligomerization in histone localization. (A) Schematic diagrams of full-length DBP and the C-terminally deleted mutant DBPΔC. DBP of HAdV5 consists of 529 aa, and the C-terminal 17 aa (aa 513 to 529) function as an "arm" for oligomerization. DBPΔC lacks the C-terminal 17 aa. (B) Western blot analyses. HeLa cells stably expressing histone H3.3-EGFP were transfected with the pCHA-puro empty vector (lane 1), pCHA-puro-DBP (lane 2), or pCHA-puro-DBPΔC (lane 3), and lysates prepared at 36 hpt were subjected to 10% SDS-PAGE, followed by Western blot analyses using anti-HA (top), anti-DBP (middle), and anti-β-actin (bottom) antibodies. (C) IF analyses. At 36 hpt, cells grown on coverslips were subjected to IF analyses using anti-HA (left) and anti-DBP (right) antibodies (B). Higher-magnification images of the regions marked by squares are shown below.

in the absence of any viral proteins/DNA, and histone H3.3-EGFP was excluded from these foci, as observed for infected cells (HA-DBP) (Fig. 6C). In sharp contrast, DBPΔC was localized throughout the nucleus and did not form such foci (Fig. 6C). Taken together, these results suggest that the oligomerization of DBP has a critical role in histone exclusion from VDRF.

DISCUSSION

In this study, we showed that replication-independent histone variant H3.3 is deposited onto both incoming and newly synthesized Ad DNA (Fig. 3). These results, together with the results from knockdown experiments with CAF-1 (Fig. 2) and microscopic analyses (Fig. 4 to 6), indicated that the histone deposition onto the replicated virus genome is most likely uncoupled with viral DNA replication. Based on these results, together with data from our previous work (20), we hypothesize a model with respect to the fluctuation of the viral chromatin structure during the infection cycle (Fig. 7). In virions, viral DNA is tightly packed with viral core proteins (13). After entry into the cell, cellular histones are incorporated into incoming viral DNA-protein VII complexes in the nucleus, and viral chromatin composed of both protein VII and histones functions as the template for viral early gene expression (20). In this process, histone H3.3 is specifically deposited onto viral DNA, possibly by a histone chaperone, HIRA (34). As infection proceeds and viral DNA replication is then initiated, the oligomerization of DBP establishes a "histone-free" environment for viral DNA replication. Newly synthesized viral DNA is then associated with histone H3.3 in a replication-uncoupling fashion and might be acting as the template for viral late gene expression outside VDRF (31). In later phases of infection (~24 hpi), both histones and newly synthesized core proteins VII and V are associated with viral DNA, which likely reflects processes during progeny virion assembly (35). Since histones are not included in virions, histones must be removed and replaced with newly synthesized core proteins for progeny virions. Although the packaging mechanism of progeny viral DNA during virion assembly remains unclear, we have reported the involvement of nucleolar protein B23/nucleophosmin in the regulation of the viral chromatin structure during progeny virion assembly (35, 36).

The mechanistic details of the histone deposition after viral

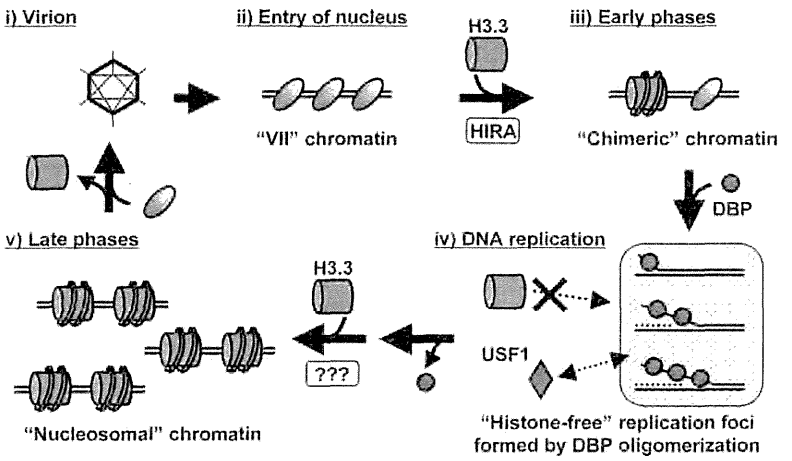

FIG 7 Hypothetical model for the viral chromatin structure during the infection cycle. For details, see Discussion.

DNA replication still remain unclear. First, what factor(s) is involved in histone deposition at late phases of infection? HIRA is a potential candidate for this process as well as in early phases of infection (34). However, we did not perform knockdown experiments for HIRA, since even if we could observe some effect of an HIRA knockdown on viral chromatin in late phases of infection, we could hardly distinguish whether the knockdown affects the chromatin structure of progeny viral DNA directly or whether the effect is derived indirectly from earlier events on incoming viral chromatin. IF analyses showed that the localization of HIRA was not drastically changed during the infection cycle (Fig. 5A). Recent reports indicated that Daxx, a component of PML bodies, is also an H3.3-specific histone chaperone (10, 21). However, Daxx seems not to function in the H3.3 deposition onto viral DNA, because during Ad infection, some components of PML bodies, including Daxx, are relocalized by the viral protein E4orf3, possibly for the inactivation of the components (6, 42). Indeed, it was shown previously that the Daxx-mediated antiviral response is antagonized by E4orf3 (48). It was also revealed that Daxx negatively functions and undergoes E1B-55K- and proteasome-dependent degradation during Ad infection (39). Furthermore, most recently, Schreiner et al. reported that during or immediately after the nuclear import of the incoming virus genome, protein VI, one of the capsid proteins, binds to and counteracts Daxx, at least partly by displacing it from PML bodies (38). Those reports strongly suggest that Daxx is inactivated entirely throughout the infection cycle by viral proteins. DEK was also recently reported to be a chaperone for histone H3.3 in *Drosophila* cells (37), but it is unknown whether human DEK also functions as a variant-specific chaperone or not. Our IF analyses indicated that exogenously expressed DEK is excluded from VDRF (Fig. 5B and C). Further studies are needed to elucidate the functions of these factors in late phases of infection.

In this study, we could not observe a role of CAF-1 in histone deposition onto viral DNA, while the accumulation of CAF-1 at VDRF was observed (Fig. 2 and 4A). The CAF-1 knockdown did not affect the binding levels of histone H3 on viral chromatin (Fig. 2C). Although we could not exclude the possibility that the knockdown efficiency of CAF-1 is not sufficient under the conditions employed here, we concluded that the function of CAF-1 is largely inhibited under our conditions: we observed that siCAF-1-treated cells exhibit aberrant cell shapes (data not shown) and that the knockdown affected viral gene expression (see below). Second, histone H3.3 is selectively incorporated into viral chromatin (Fig. 3), while CAF-1 generally functions as a chaperone for H3.1 and H3.2 (43). Third, although CAF-1 was reported previously to be able to be associated with H3.3 under some specific conditions (10, 21), we could not observe any interaction between CAF-1 and H3.3 during Ad infection, at least under our experimental conditions (data not shown). In addition to their roles during DNA replication, CAF-1 and PCNA were also reported previously to be involved in the DNA damage response pathway (29). Carson et al. reported that the DNA damage response pathway is only partially activated during Ad infection and that some related factors, such as ATRIP and TopBP1, are accumulated at VDRF (5). Therefore, CAF-1 (and PCNA) might localize at VDRF in the course of this limited DNA damage response. Recently, it was reported that FANCD2, one of the factors involved in the DNA damage response, is accumulated at VDRF and that the loss of this protein results in lower expression levels of viral late, but not early, genes (8). Similarly, we observed that the CAF-1 knockdown affected mRNA levels of viral late genes without any effect on viral DNA replication (our unpublished observations). Thus, factors related to the DNA damage response, such as FANCD2 and CAF-1, might be required for viral late gene expression, although the underlying mechanisms are unknown. Under our conditions, the CAF-1 knockdown did not affect the binding level of histone H3 on cellular chromatin (rRNA gene) (Fig. 2C). This is consistent with a previous report that the loss of CAF-1 impairs the replication-coupled deposition of histones but that the formation of nucleosome arrays on genomic DNA was still observed in the absence of CAF-1 (44). In addition, a recent report demonstrated that a defect of histone H3.1 deposition by CAF-1 depletion could be rescued by HIRA-mediated H3.3 deposition (33). Thus, in the case of cellular chromatin, an alternative histone deposition pathway(s) could rescue the loss of CAF-1 function.

It remains to be clarified what is the biological/virological significance of histone deposition uncoupled with viral DNA replication. On cellular chromatin, a replication-dependent histone chaperone, CAF-1, is associated with the DNA replication ma-

chinery and deposits histone H3.1-H4 (and H3.2-H4) complexes during DNA replication (14, 40, 43). This DNA replication-coupled system of histone deposition is thought to also be utilized by some DNA viruses. For instance, the DNA replication of simian virus 40 (SV40) is dependent largely on the cellular replication machinery, and indeed, CAF-1 was originally identified by using cell-free DNA replication systems of SV40 (41). For cytomegalovirus infection, it was reported previously that cellular histones, CAF-1, and PCNA accumulated at viral replication compartments (25). In the case of herpes simplex virus 1, it was shown that histone H3.3 is first deposited onto incoming viral DNA by HIRA and that H3.1 then becomes associated with viral DNA accompanied by viral DNA replication (28). It was suggested that this functional link between DNA replication and histone deposition enables the transfer of "epigenetic memory," such as histone modifications, to the daughter DNA strands (43). Thus, some DNA viruses might take advantage of this system for late gene expression, which generally occurs after viral DNA replication. On the other hand, Ad seems to utilize another strategy, that is, the uncoupling mechanism, as shown here. Like other DNA viruses, Ad late genes are expressed only after the onset of viral DNA replication. Thomas and Mathews demonstrated previously that Ad late gene expression requires its DNA replication in *cis* (45), although the molecular mechanism remains to be determined. This report leads us to hypothesize that the regulation of the viral chromatin structure during DNA replication could be an important process for late gene expression. In general, the histone/nucleosome structure on DNA restricts the access of *trans*-acting factors, such as transcription factors. In this view, DBP is an attractive candidate for the key regulatory factor for the DNA replication-dependent expression of viral late genes. By oligomerization, DBP is able to not only support viral DNA replication but also establish the "histone-free" environment, which could be an opportunity window for transcription factors to access the viral DNA for the activation of viral late genes. Our IF analyses showed that the transcription factor USF1, which binds to the MLP region after viral DNA replication (46), is not excluded from VDRF (Fig. 5B and C), supporting this notion. Furthermore, this is in agreement with a previous report that DBP enhances the binding of USF1 to the MLP region *in vitro* (51). Overall, we speculate that the uncoupling of histone deposition with viral DNA replication is mediated by DBP oligomerization, at least partly, and plays a role in the DNA replication-dependent activation of viral late gene expression.

The expression of certain cellular genes, such as the *HoxB* gene, has been shown to require DNA replication (12). However, the regulation mechanism of "DNA replication-dependent gene expression" remains to be determined. As Ad has late genes, the expressions of which are DNA replication dependent (45), this virus could be a good model for analyses of such regulations. Therefore, this study might give a clue to an understanding of the functional relationship between DNA replication and transcription on cellular and/or viral chromatin.

ACKNOWLEDGMENTS

We thank M. Okuwaki, S. Saito, A. Verreault, W. C. Russel, and K. Kajitani for their kind gifts.

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K.N.) and the University of Tsukuba research infrastructure support program (to T.K.).

REFERENCES

1. **Ahmad K, Henikoff S.** 2002. The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol. Cell **9**:1191–1200.
2. **Bell O, Tiwari VK, Thoma NH, Schubeler D.** 2011. Determinants and dynamics of genome accessibility. Nat. Rev. Genet. **12**:554–564.
3. **Beyer AL, Bouton AH, Hodge LD, Miller OL, Jr.** 1981. Visualization of the major late R strand transcription unit of adenovirus serotype 2. J. Mol. Biol. **147**:269–295.
4. **Burg JL, Schweitzer J, Daniell E.** 1983. Introduction of superhelical turns into DNA by adenoviral core proteins and chromatin assembly factors. J. Virol. **46**:749–755.
5. **Carson CT, et al.** 2009. Mislocalization of the MRN complex prevents ATR signaling during adenovirus infection. EMBO J. **28**:652–662.
6. **Carvalho T, et al.** 1995. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. J. Cell Biol. **131**:45–56.
7. **Chatterjee PK, Vayda ME, Flint SJ.** 1986. Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. EMBO J. **5**:1633–1644.
8. **Cherubini G, et al.** 2011. The FANC pathway is activated by adenovirus infection and promotes viral replication-dependent recombination. Nucleic Acids Res. **39**:5459–5473.
9. **Dekker J, et al.** 1997. Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis. EMBO J. **16**:1455–1463.
10. **Drane P, Ouararhni K, Depaux A, Shuaib M, Hamiche A.** 2010. The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. Genes Dev. **24**:1253–1265.
11. **Elsaesser SJ, Goldberg AD, Allis CD.** 2010. New functions for an old variant: no substitute for histone H3.3. Curr. Opin. Genet. Dev. **20**:110–117.
12. **Fisher D, Mechali M.** 2003. Vertebrate HoxB gene expression requires DNA replication. EMBO J. **22**:3737–3748.
13. **Giberson AN, Davidson AR, Parks RJ.** 2012. Chromatin structure of adenovirus DNA throughout infection. Nucleic Acids Res. **40**:2369–2376. doi:10.1093/nar/gkr1076.
14. **Groth A, Rocha W, Verreault A, Almouzni G.** 2007. Chromatin challenges during DNA replication and repair. Cell **128**:721–733.
15. **Gyurcsik B, Haruki H, Takahashi T, Mihara H, Nagata K.** 2006. Binding modes of the precursor of adenovirus major core protein VII to DNA and template activating factor I: implication for the mechanism of remodeling of the adenovirus chromatin. Biochemistry **45**:303–313.
16. **Hake SB, Allis CD.** 2006. Histone H3 variants and their potential role in indexing mammalian genomes: the "H3 barcode hypothesis." Proc. Natl. Acad. Sci. U. S. A. **103**:6428–6435.
17. **Haruki H, Gyurcsik B, Okuwaki M, Nagata K.** 2003. Ternary complex formation between DNA-adenovirus core protein VII and TAF-Ibeta/SET, an acidic molecular chaperone. FEBS Lett. **555**:521–527.
18. **Haruki H, Okuwaki M, Miyagishi M, Taira K, Nagata K.** 2006. Involvement of template-activating factor I/SET in transcription of adenovirus early genes as a positive-acting factor. J. Virol. **80**:794–801.
19. **Kawase H, et al.** 1996. NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. Genes Cells **1**:1045–1056.
20. **Komatsu T, Haruki H, Nagata K.** 2011. Cellular and viral chromatin proteins are positive factors in the regulation of adenovirus gene expression. Nucleic Acids Res. **39**:889–901.
21. **Lewis PW, Elsaesser SJ, Noh KM, Stadler SC, Allis CD.** 2010. Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. Proc. Natl. Acad. Sci. U. S. A. **107**:14075–14080.
22. **Matsumoto K, Nagata K, Ui M, Hanaoka F.** 1993. Template activating factor I, a novel host factor required to stimulate the adenovirus core DNA replication. J. Biol. Chem. **268**:10582–10587.
23. **Matsumoto K, et al.** 1995. Stimulation of DNA transcription by the replication factor from the adenovirus genome in a chromatin-like structure. J. Biol. Chem. **270**:9645–9650.
24. **Nagata K, et al.** 1995. Replication factor encoded by a putative oncogene,

set, associated with myeloid leukemogenesis. Proc. Natl. Acad. Sci. U. S. A. 92:4279–4283.
25. Nitzsche A, Paulus C, Nevels M. 2008. Temporal dynamics of cytomegalovirus chromatin assembly in productively infected human cells. J. Virol. 82:11167–11180.
26. Okuwaki M, Iwamatsu A, Tsujimoto M, Nagata K. 2001. Identification of nucleophosmin/B23, an acidic nucleolar protein, as a stimulatory factor for in vitro replication of adenovirus DNA complexed with viral basic core proteins. J. Mol. Biol. 311:41–55.
27. Okuwaki M, Nagata K. 1998. Template activating factor-I remodels the chromatin structure and stimulates transcription from the chromatin template. J. Biol. Chem. 273:34511–34518.
28. Placek BJ, et al. 2009. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. J. Virol. 83:1416–1421.
29. Polo SE, Roche D, Almouzni G. 2006. New histone incorporation marks sites of UV repair in human cells. Cell 127:481–493.
30. Polo SE, et al. 2004. Chromatin assembly factor-1, a marker of clinical value to distinguish quiescent from proliferating cells. Cancer Res. 64:2371–2381.
31. Pombo A, Ferreira J, Bridge E, Carmo-Fonseca M. 1994. Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. EMBO J. 13:5075–5085.
32. Ray-Gallet D, et al. 2002. HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. Mol. Cell 9:1091–1100.
33. Ray-Gallet D, et al. 2011. Dynamics of histone h3 deposition in vivo reveal a nucleosome gap-filling mechanism for h3.3 to maintain chromatin integrity. Mol. Cell 44:928–941.
34. Ross PJ, et al. 2011. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. J. Virol. 85:3950–3958.
35. Samad MA, Komatsu T, Okuwaki M, Nagata K. 15 February 2012, posting date. B23/nucleophosmin is involved in regulation of adenovirus chromatin structure at late infection stages, but not in its replication and transcription. J. Gen. Virol. [Epub ahead of print.] doi:10.1099/vir.0.036665-0.
36. Samad MA, Okuwaki M, Haruki H, Nagata K. 2007. Physical and functional interaction between a nucleolar protein nucleophosmin/B23 and adenovirus basic core proteins. FEBS Lett. 581:3283–3288.
37. Sawatsubashi S, et al. 2010. A histone chaperone, DEK, transcriptionally coactivates a nuclear receptor. Genes Dev. 24:159–170.
38. Schreiner S, et al. 2012. Transcriptional activation of the adenoviral genome is mediated by capsid protein VI. PLoS Pathog. 8:e1002549. doi:10.1371/journal.ppat.1002549.
39. Schreiner S, et al. 2010. Proteasome-dependent degradation of Daxx by the viral E1B-55K protein in human adenovirus-infected cells. J. Virol. 84:7029–7038.
40. Shibahara K, Stillman B. 1999. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell 96:575–585.
41. Smith S, Stillman B. 1989. Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. Cell 58:15–25.
42. Stracker TH, et al. 2005. Serotype-specific reorganization of the Mre11 complex by adenoviral E4orf3 proteins. J. Virol. 79:6664–6673.
43. Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y. 2004. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell 116:51–61.
44. Takami Y, Ono T, Fukagawa T, Shibahara K, Nakayama T. 2007. Essential role of chromatin assembly factor-1-mediated rapid nucleosome assembly for DNA replication and cell division in vertebrate cells. Mol. Biol. Cell 18:129–141.
45. Thomas GP, Mathews MB. 1980. DNA replication and the early to late transition in adenovirus infection. Cell 22:523–533.
46. Toth M, Doerfler W, Shenk T. 1992. Adenovirus DNA replication facilitates binding of the MLTF/USF transcription factor to the viral major late promoter within infected cells. Nucleic Acids Res. 20:5143–5148.
47. Tucker PA, et al. 1994. Crystal structure of the adenovirus DNA binding protein reveals a hook-on model for cooperative DNA binding. EMBO J. 13:2994–3002.
48. Ullman AJ, Hearing P. 2008. Cellular proteins PML and Daxx mediate an innate antiviral defense antagonized by the adenovirus E4 ORF3 protein. J. Virol. 82:7325–7335.
49. Vayda ME, Rogers AE, Flint SJ. 1983. The structure of nucleoprotein cores released from adenovirions. Nucleic Acids Res. 11:441–460.
50. Verreault A, Kaufman PD, Kobayashi R, Stillman B. 1996. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. Cell 87:95–104.
51. Zijderveld DC, d'Adda di Fagagna F, Giacca M, Timmers HT, van der Vliet PC. 1994. Stimulation of the adenovirus major late promoter in vitro by transcription factor USF is enhanced by the adenovirus DNA binding protein. J. Virol. 68:8288–8295.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 30 January 2012
doi: 10.3389/fmicb.2012.00014

Cell tropism and pathogenesis of measles virus in monkeys

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Measles virus (MV) is an enveloped negative strand RNA virus belonging to the family of Paramyxoviridae, genus *Morbillivirus*, and causes one of the most contagious diseases in humans. Experimentally infected non-human primates are used as animal models for studies of the pathogenesis of human measles. We established a reverse genetics system based on a highly pathogenic wild-type MV. Infection of monkeys with recombinant MV strains generated by reverse genetics enabled analysis of the molecular basis of MV pathogenesis. The essential *in vivo* function of accessory genes was indicated by infecting monkeys with recombinant MV strains deficient in the expression of accessory genes. Furthermore, recombinant wild-type MV strains expressing enhanced green fluorescent protein enabled visual tracking of MV-infected cells *in vitro* and *in vivo*. To date, three different molecules have been identified as receptors for MV. Signaling lymphocyte activation molecule (SLAM, also called CD150), expressed on immune cells, is a major receptor for MV. CD46, ubiquitously expressed in all nucleated cells in humans and monkeys, is a receptor for vaccine and laboratory-adapted strains of MV. The newly identified nectin-4 (also called poliovirus-receptor-like-4) is an epithelial cell receptor for MV. However, recent findings have indicated that CD46 acts as an MV receptor *in vitro* but not *in vivo*. The impact of the receptor usage of MV *in vivo* on the disease outcome is now under investigation.

Keywords: measles virus, monkey, pathogenesis, tropism, reverse genetics, receptor, EGFP

INTRODUCTION

Measles is a febrile disease that typically occurs in small children; the incubation period is 10–14 days, after which clinical symptoms such as fever, coughing, and a characteristic rash appears. Since measles is accompanied by immunosuppression, it has a high frequency of complication with secondary bacterial infections, such as otitis media or pneumonia. Although developed countries are eradicating measles by promoting effective vaccination, measles remains an important issue, especially in developing countries (Griffin, 2007).

Measles virus (MV), belonging to the genus *Morbillivirus* of the family Paramyxoviridae, is an enveloped virus with a non-segmented negative strand RNA genome. The MV genome has six genes that encode the nucleocapsid (N), phospho (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins (**Figure 1A**). MV contains two envelope glycoproteins: the H protein, which is responsible for receptor binding and is important for determining cell tropism of MV; and the F protein, which mediates membrane fusion (Navaratnarajah et al., 2009). The P gene encodes the P protein and the non-structural V and C proteins. The V and C proteins are important for antagonizing the host interferon (IFN) response (Gerlier and Valentin, 2009).

To date, three different molecules have been identified as receptors for MV. Signaling lymphocyte activation molecule (SLAM, also called CD150), expressed in certain immune system cells including activated B and T lymphocytes, mature dendritic cells, and macrophages, is a receptor for wild-type MV and vaccine and laboratory-adapted strains of MV (Tatsuo et al., 2000). CD46 (also called membrane cofactor protein), expressed in all human and monkey nucleated cells, is a receptor for vaccine and laboratory-adapted strains of MV (Dorig et al., 1993; Naniche et al., 1993). Recently, nectin-4 [also called poliovirus-receptor-like-4 (PVRL4)] has been identified as the epithelial receptor for wild-type MV (Muhlebach et al., 2011; Noyce et al., 2011).

Several animal models have been used for studying the pathogenesis of MV (Griffin, 2007). Cotton rats, rats, hamsters, mice, and ferrets can be infected with MV and are commonly used as small animal models for MV pathogenesis. After identification of CD46 and SLAM as MV receptors, numerous transgenic and knock-in mice expressing human CD46 and/or SLAM were established and intensively used to study different aspects of MV infection (Sellin and Horvat, 2009). However, non-human primates are the only animals that exhibit acute disease similar to that seen in humans. In this review, we discuss recent findings regarding tropism and pathogenesis of MV; these findings were obtained by infecting monkeys with recombinant wild-type MV.

HISTORICAL BACKGROUND OF MONKEY MODELS

When infected with measles, monkeys exhibit similar symptoms as seen in humans. This was reported as early as 1911, after inoculating monkeys with blood from measles patients (Anderson and Goldberger, 1911). In 1921, it was reported that measles could be transmitted from humans to monkeys by placing a filtered throat swab from a measles patient into the tracheae of monkeys (Blake and Trask, 1921a). These authors also performed histological analysis of infected monkeys (Blake and Trask, 1921b). However,

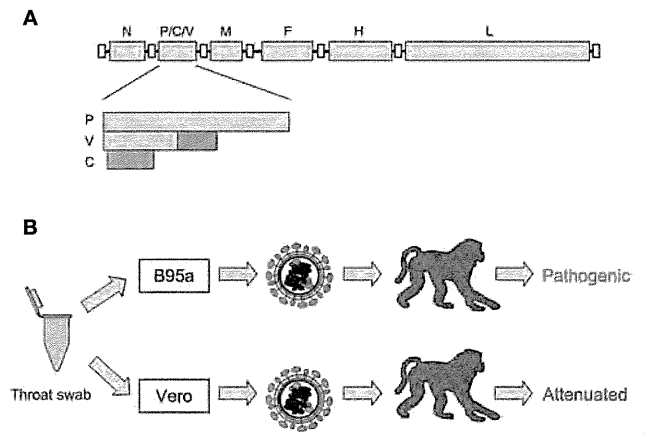

FIGURE 1 | (A) Schematic diagram of measles virus genome. The P gene encodes the P protein and the non-structural V and C proteins. The V protein is translated from an edited RNA with a non-templated G residue. The V protein-specific region is indicated in red. The C protein is translated from an alternative open reading frame (purple) initiated by the second AUG. **(B)** Isolation of wild-type MV using B95a cells. Pathogenic wild-type MV strains can be isolated by inoculating throat swabs from measles patients in B95a cells. MV strains isolated in Vero cells lose their pathogenicity.

at this time, "MV" had not yet been discovered. MV was first isolated in 1954 from a specimen obtained from a measles patient (Enders and Peebles, 1954). Enders and Peebles (1954) inoculated human and monkey cell cultures with a throat swab taken from a young boy named David Edmonston and isolated MV from these cultures. After this, it was discovered that MV isolated from normal human renal cells caused clinical signs similar to those of human measles in monkeys (Peebles et al., 1957). Since then, numerous studies have been carried out by infecting monkeys with MV, measles vaccines, or specimens from measles patients (Griffin, 2007). In such experiments, two species of monkeys, cynomolgus monkey (*Macaca fascicularis*) and rhesus monkey (*Macaca mulatta*), serve as good animal models. New World monkeys are more susceptible to MV than Old World monkeys, and infection of marmoset (*Saguinus mystax*) with MV results in a fulminant disease (Levy and Mirkovic, 1971; Albrecht et al., 1980).

In the past, it was well known that infection of monkeys with materials from measles patients induced clinical signs similar to those of human measles (Nii et al., 1964; Yamanouchi et al., 1970; Sakaguchi et al., 1986). However, curiously enough, infection of monkeys with MV isolated and propagated in cultured cells did not always induce these clinical signs (Enders et al., 1960; Yamanouchi et al., 1970; van Binnendijk et al., 1994). This riddle was solved by the introduction of B95a cells (a marmoset B-lymphoid cell line) for isolation and propagation of MV (Kobune et al., 1990). Kobune et al. (1990, 1996) found that MV strains could be efficiently isolated in B95a cells using materials from measles patients. More importantly, MV strains isolated from B95a cells retained their original pathogenicity in monkeys. These studies indicated that vaccine and laboratory-adapted strains of MV previously isolated from non-lymphoid cells such as Vero cells were not true MV (**Figure 1B**). A decade later, it was found that the MV receptor SLAM is highly expressed on B95a cells (Tatsuo et al., 2000), which accounts for the efficient isolation of pathogenic MV from patient samples. Similar to MV strains isolated from B95a cells, MV strains isolated and propagated in monkey mononuclear cells, human cord blood cells, human B lymphoblastoid cell lines, and Vero cells expressing SLAM replicated well in monkeys and induced clinical signs of measles (van Binnendijk et al., 1994; McChesney et al., 1997; Zhu et al., 1997; Auwaerter et al., 1999; El Mubarak et al., 2007; Bankamp et al., 2008). These results suggest that expression of SLAM on cells used for isolation is important for isolation of pathogenic MV.

REVERSE GENETICS OF MV

Reverse genetics refers to the methods used for recovering infectious viruses from the cDNA that encodes the viral genome. By using this method, mutations or extra transcription units can be introduced into viral genomes by the modification of cDNA plasmids. Reverse genetics of MV was first established based on the Edmonston vaccine strain (Radecke et al., 1995). However, as previously mentioned, viruses derived from the Edmonston vaccine strain do not induce clinical symptoms of measles in monkeys. Therefore, reverse genetics of pathogenic wild-type MV was needed for the study of MV pathogenesis in monkeys. To this end, we first determined the complete nucleotide sequence of the genome of the pathogenic wild-type IC-B strain (NC_001498/AB016162; Takeuchi et al., 2000), which was isolated in Tokyo in 1984 by using B95a cells (Kobune et al., 1990). Then, we constructed a complete cDNA plasmid of the IC-B strain named p(+)MV323, and successfully recovered infectious MV (IC323 strain) from the p(+)MV323 plasmid (Takeda et al., 2000). Importantly, the IC323 strain induced clinical signs such as rash, Koplik's spots, and lymphopenia similar to human measles in infected monkeys, indicating that the IC323 strain retains the original pathogenicity of the IC-B strain. Now, infectious MV strains can be easily recovered from cDNA plasmids by using an improved protocol (Takeda et al., 2005).

Reverse genetics of other wild-type MV strains has been reported for the HL strain isolated in Japan (Terao-Muro et al., 2008) and the KS strain isolated in Sudan (Lemon et al., 2011). For vaccine strains of MV, reverse genetics for the Schwarz/Moraten vaccine strain (Combredet et al., 2003; del Valle et al., 2007) and the AIK-C vaccine strain (Nakayama et al., 2001) have also been reported. Reverse genetics for vaccine strains are being used as a platform to generate new multivalent vaccines expressing antigens of other pathogens (Billeter et al., 2009) and oncolytic viruses for cancer therapy (Russell and Peng, 2009).

FUNCTION OF MV ACCESSORY PROTEINS *IN VIVO*

The P gene of MV encodes two non-structural proteins, namely the C and V proteins. However, the function of the C and V proteins *in vivo* was not well understood. The C protein is a small (186 amino acid), highly positively charged protein. To study the function of the C protein in the context of the natural course of MV pathogenesis, we generated an IC323 strain deficient in the expression of the C protein, wtMV(C−), by using the reverse genetics of wild-type MV (Takeuchi et al., 2005). Notably, the growth of wtMV(C−) in cynomolgus monkeys was dramatically reduced

when compared to the IC323 strain. A similar growth defect of the IC323 strain deficient in the expression of the C protein, C^{ko}, *in vivo* was observed in rhesus monkeys (Devaux et al., 2008). Interestingly, C^{ko} induced more inflammatory cytokines such as tumor necrosis factor alpha (TNF)-α and interleukin (IL)-6 and interferon (IFN)-α and -β in infected monkeys.

The V protein is translated from an edited mRNA of the P gene (Griffin, 2007). Thus, the amino-terminal domain of the V protein has the same amino acid sequence as the P protein, and the carboxyl-terminal domain of the V protein has a highly conserved amino acid sequence forming a zinc-binding domain, which is important for function as an interferon antagonist (Gerlier and Valentin, 2009). An IC323 strain deficient in the expression of the V protein, V^{ko}, was generated by introducing nucleotide mutations in the RNA-editing signal in the P gene (Devaux et al., 2008). The growth of V^{ko} in infected rhesus monkeys was lower than that of the parental IC323 strain. V^{ko} induced more inflammatory cytokines (TNF-α and IL-6) and IFN-α and -β. An IC323 strain unable to antagonize STAT1 function, STAT1-blind virus, was generated by introducing three amino acid substitutions in the shared domain of the P and V proteins (Devaux et al., 2011). The STAT1-blind virus induced short-lived viremia and no clinical signs in infected rhesus monkeys. This virus induced more inflammatory cytokines (TNF-α and IL-6) and a Th1/Th2 balance cytokine (IL-12) in infected monkeys. Taken together, these findings indicate that the C and V proteins are not non-essential gene products as previously thought, but are stringently required for antagonizing host innate immune and inflammatory responses *in vivo*.

In contrast, *in vitro* studies indicated that the V protein blocks IFN-α/β signal transductions in infected cells, inhibits TLR7-mediated IFN-α production in human plasmacytoid dendritic cells, and inhibits IFN induction in infected cells by interacting with MDA5 (Gerlier and Valentin, 2009). Furthermore, the C protein appears to inhibit IFN induction in infected cells by regulating viral RNA synthesis (Nakatsu et al., 2008). Thus, it is necessary to elucidate whether the *in vivo* phenotypes of C- and V-deficient viruses are similar to the *in vitro* phenotypes.

MV TROPISM *IN VIVO*

Recent advances in the study of virus tropism have included the introduction of enhanced green fluorescent protein (EGFP)-expressing viruses. *In vivo* tropism of *Morbillivirus* can be visualized with high sensitivity in living animals as well as tissue samples by using EGFP-expressing recombinant canine distemper viruses (von Messling et al., 2004). Similarly, MV target tissues or organs can be visualized with high sensitivity by infecting cynomolgus monkeys with an EGFP-expressing IC323 strain (**Figure 2**). de Swart et al. (2007) infected rhesus and cynomolgus monkeys with an IC323 strain expressing EGFP (Hashimoto et al., 2002) and examined the tropism of wild-type MV *in vivo*. They indicated that the major target cells of wild-type MV were B and T lymphocytes and CD11c-positive, major histocompatibility complex (MHC) class-II-positive dendritic cells. This result is consistent with the fact that SLAM is a receptor for wild-type MV. Infection of ciliated epithelial cells in the trachea and lungs was also detected, suggesting the presence of another receptor for MV in epithelial cells.

Regarding the early target cells of wild-type MV, classical textbooks describe that the primary targets of MV are the epithelial cells of the respiratory tract. However, SLAM is not expressed in these epithelial cells. To examine the early target cells of wild-type and vaccine strains of MV in the lung, de Vries et al. (2010) infected cynomolgus monkeys with EGFP-expressing IC323 or vaccine strains of MV via the intratracheal or aerosol route. They found

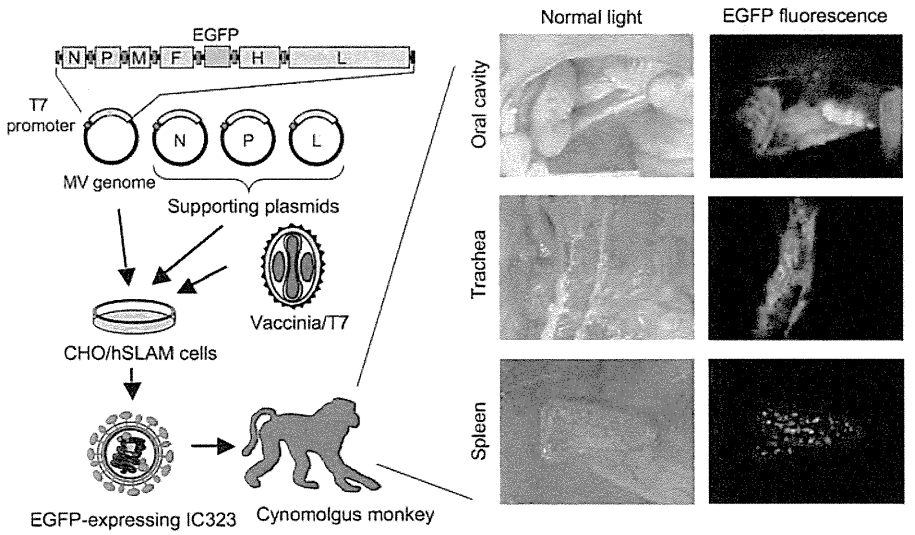

FIGURE 2 | Schematic diagram of reverse genetics of wild-type MV expressing EGFP and infection of monkeys. A plasmid carrying the full-genome cDNA of the IC-B strain and the EGFP gene under the control of the T7 promoter is introduced into CHO cells expressing human SLAM (CHO/hSLAM), along with three supporting plasmids expressing N, P, and L proteins, respectively, under the control of the T7 promoter. After infection with vaccinia virus expressing the T7 RNA polymerase, wild-type MV expressing EGFP can be recovered by mixing with B95a cells. EGFP fluorescence in tissues and organs of infected monkeys can be detected using a fluorescence microscope.

that CD11c-positive cells, which include alveolar macrophages and dendritic cells, were the major targets of both viruses. Interestingly, although viral replication and cellular tropism in the lungs were similar for the two viruses, only wild-type MV caused significant viremia, suggesting a growth defect of the vaccine strain in lymphocyte cells. Similarly, to examine the early target cells of wild-type MV, Lemon et al. (2011) infected cynomolgus monkeys with an EGFP-expressing wild-type MV based on the KS strain by aerosol infection and found that the early target cells of wild-type MV in monkeys are macrophages and dendritic cells. These studies indicated that alveolar macrophages and dendritic cells but not the epithelial cells of the respiratory tract are the early target cells of wild-type MV.

Nectin-4 is a newly identified epithelial cell receptor (EpR) for MV. To examine the effect of nectin-4-using activity of MV on disease outcomes in monkeys, an IC323 strain recognizing SLAM but not nectin-4 was generated by introducing amino acid mutations in the H protein (Leonard et al., 2008). At that time, nectin-4 had not been identified as a MV receptor, and this strain was called EpR-blind virus. When rhesus monkeys were infected with EpR-blind virus via the conjunctiva and nares, this virus induced viremia and clinical signs in infected monkeys but did not propagate in the lungs. This result indicates the importance of nectin-4 for the propagation of MV in the lungs, which is required for the subsequent exit of MV from the host. Inversely, to examine the impact of the recognition of SLAM by MV, an IC323 strain recognizing nectin-4 but not SLAM, SLAM-blind, was generated (Leonard et al., 2010). When rhesus monkeys were infected with the SLAM-blind virus, it elicited no clinical symptoms. This result indicates that SLAM recognition is necessary for MV virulence and pathogenesis.

Vaccine and laboratory-adapted strains of MV can utilize both CD46 and SLAM as cellular receptors. However, surprisingly, the impact of the CD46-using activity of vaccine and laboratory-adapted strains of MV on their tissue and organ tropism and attenuation is not well understood. As CD46 is ubiquitously expressed on all nucleated human and monkey cells, vaccine, and laboratory-adapted strains of MV may infect all tissues and organs of humans and monkeys. If so, this tropism shift may have great consequences on vaccine attenuation. In this context, de Vries et al. (2010) indicated that only CD11c-positive cells were infected with the EGFP-expressing vaccine strain via the aerosol route, suggesting that vaccine strains do not use CD46 *in vivo*. However, when the replication of vaccine and laboratory-adapted strains of MV in monkeys is limited, it will be difficult to identify infected cells in tissues. Furthermore, the infection of vaccine and laboratory-adapted strains of MV may be restricted because of mutations in the P/C/V genes, which are important for antagonizing the host IFN response (Gerlier and Valentin, 2009). Therefore, the tropism shift that solely occurs via the H protein should be evaluated using the wild-type MV expressing EGFP, which bears the H protein of a vaccine strain, such as the IC/EdH strain we developed previously (Takeuchi et al., 2002).

We recently infected cynomolgus monkeys with EGFP-expressing wild-type or IC/EdH strains and found that SLAM-expressing lymphocytes were the main targets of both strains, indicating that CD46 does not act as a receptor for vaccine and laboratory-adapted strains of MV *in vivo* (**Figure 3**; Takeuchi et al., 2012). One possible explanation for the limited expansion of the EGFP-expressing IC/EdH strain *in vivo* is the activation status of lymphocytes. It is known that stimulated lymphocytes are efficiently infected with MV and that stimulated lymphocytes express SLAM (Tatsuo et al., 2000). Thus, lymphocytes expressing SLAM may appear to be infected with both strains. The EGFP-expressing IC/EdH strain that entered into quiescent lymphocytes via the CD46 may not grow well in those cells. Alternatively, the expression level of CD46 *in vivo* may be too low to allow MV dissemination by cell–cell fusion, as it was reported that high CD46 density is required for MV-induced cell–cell fusion (Anderson et al., 2004). For whatever the reason, the growth of the EGFP-expressing IC/EdH strain was less efficient than that of the EGFP-expressing wild-type strain (Takeuchi et al., 2012), suggesting that the CD46-recognition ability of vaccine and laboratory-adapted MV strains plays a role in the MV attenuation. Further studies are required to elucidate the relationship between CD46-recognition ability and MV attenuation.

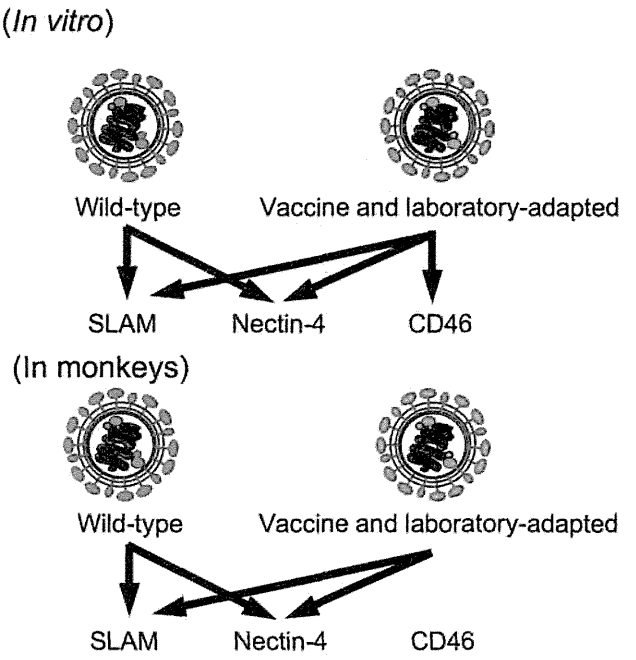

FIGURE 3 | Receptor usage of the wild-type and vaccine and laboratory-adapted strains of MV. Nectin-4 is a recently identified epithelial cell receptor for MV.

CONCLUSION

As described in this review, our IC323 strain, recovered from a plasmid carrying the full-genome cDNA of the IC-B strain, is now used as a standard wild-type MV strain in MV research. Furthermore, EGFP-expressing IC323 strains are ideal tools for the study of tissue and organ tropism of wild-type MV *in vivo*. Although the use of monkeys has several limitations, monkey models still provide the only reliable animal model for the study of the pathogenesis of MV in humans. The combination of wild-type and vaccine

MV strains generated by reverse genetics and monkey models will provide new insights into the relationship between viral gene functions or individual mutation(s) and the pathogenesis of MV.

ACKNOWLEDGMENTS

This paper was supported by a grant-in-aid (No. 23659227) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

Albrecht, P., Lorenz, D., Klutch, M. J., Vickers, J. H., and Ennis, F. A. (1980). Fatal measles infection in marmosets pathogenesis and prophylaxis. *Infect. Immun.* 27, 969–978.

Anderson, B. D., Nakamura, T., Russell, S. J., and Peng, K.-W. (2004). High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus. *Cancer Res.* 64, 4919–4926.

Anderson, J. F., and Goldberger, J. (1911). Experimental measles in the monkey: a supplemental note. *Public Health Rep.* 26, 887–895.

Auwaerter, P. G., Rota, P. A., Elkins, W. R., Adams, R. J., DeLozier, T., Shi, Y., Bellini, W. J., Murphy, B. R., and Griffin, D. E. (1999). Measles virus infection in rhesus macaques: altered immune responses and comparison of the virulence of six different virus strains. *J. Infect. Dis.* 180, 950–958.

Bankamp, B., Hodge, G., McChesney, M. B., Bellini, W. J., and Rota, P. A. (2008). Genetic changes that affect the virulence of measles virus in a rhesus macaque model. *Virology* 373, 39–50.

Billeter, M. A., Naim, H. Y., and Udem, S. A. (2009). Reverse genetics of measles virus and resulting multivalent recombinant vaccines: applications of recombinant measles viruses. *Curr. Top. Microbiol. Immunol.* 329, 129–162.

Blake, F. G., and Trask, J. D. Jr. (1921a). Studies on measles. I. Susceptibility of monkeys to the virus of measles. *J. Exp. Med.* 33, 385–412.

Blake, F. G., and Trask, J. D. Jr. (1921b). Studies on measles. II. Symptomatology and pathology in monkeys experimentally infected. *J. Exp. Med.* 33, 413–422.

Combredet, C., Labrousse, V., Mollet, L., Lorin, C., Delebecque, F., Hurtrel, B., McClure, H., Feinberg, M. B., Brahic, M., and Tangy, F. (2003). A molecularly cloned Schwarz strain of measles virus vaccine induces strong immune responses in macaques and transgenic mice. *J. Virol.* 77, 11546–11554.

de Swart, R. L., Ludlow, M., de Witte, L., Yanagi, Y., van Amerongen, G., McQuaid, S., Yuksel, S., Geijtenbeek, T. B. H., Duprex, W. P., and Osterhaus, A. D. M. E. (2007). Predominant infection of CD150+ lymphocytes and dendritic cells during measles virus infection of macaques. *PLoS Pathog.* 3, e178. doi:10.1371/journal.ppat.0030178

de Vries, R. D., Lemon, K., Ludlow, M., McQuaid, S., Yuksel, S., van Amerongen, G., Rennick, L. J., Rima, B. K., Osterhaus, A. D. M. E., de Swart, R. L., and Duprex, W. P. (2010). In vitro tropism of attenuated and pathogenic measles virus expressing green fluorescent protein in macaques. *J. Virol.* 84, 4714–4724.

del Valle, J. R., Devaux, P., Hodge, G., Wegner, N. J., McChesney, M. B., and Catteneo, R. (2007). A vectored measles virus induces hepatitis B surface antigen antibodies while protecting macaques against measles virus challenge. *J. Virol.* 81, 10597–10605.

Devaux, P., Hodge, G., McChesney, M. B., and Cattaneo, R. (2008). Attenuation of V- or C-defective measles viruses: infection control by the inflammatory and interferon responses of rhesus monkeys. *J. Virol.* 82, 5359–5367.

Devaux, P., Hudacek, A. W., Hodge, G., Valle, J. R., McChesney, M. B., and Cattaneo, R. (2011). A recombinant measles virus unable to antagonize STAT1 function cannot control inflammation and is attenuated in rhesus monkeys. *J. Virol.* 85, 348–356.

Dorig, R. E., Marcil, A., Chopra, A., and Richardson, C. D. (1993). The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell* 75, 295–305.

El Mubarak, H. S., Yuksel, S., van Amerongen, G., Mulder, P. G. H., Mukhtar, M. M., Osterhaus, A. D. M. E., and de Swart, R. L. (2007). Infection of cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) with different wild-type measles viruses. *J. Gen. Virol.* 88, 2028–2034.

Enders, J. F., Katz, S. L., Milovanovic, M. V., and Holloway, A. (1960). Studies on an attenuated measles-virus vaccine I. Development and preparation of the vaccine: techniques for assay of effects of vaccination. *N. Engl. J. Med.* 263, 153–159.

Enders, J. F., and Peebles, T. C. (1954). Propagation in tissue cultures of cytopathic agents from patients with measles. *Proc. Soc. Exp. Biol. Med.* 86, 277–286.

Gerlier, D., and Valentin, H. (2009). Measles virus interaction with host cells and impact on innate immunity. *Curr. Top. Microbiol. Immunol.* 329, 163–191.

Griffin, D. E. (2007). "Measles virus," in *Fields Virology*, 5th Edn, eds D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (Philadelphia, PA: Lippincott Williams & Wilkins), 1551–1585.

Hashimoto, K., Ono, N., Tatsuo, H., Minagawa, H., Takeda, M., Takeuchi, K., and Yanagi, Y. (2002). SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. *J. Virol.* 76, 6743–6749.

Kobune, F., Sakata, H., and Sugiura, A. (1990). Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. *J. Virol.* 64, 700–705.

Kobune, F., Takahashi, H., Terao, K., Ohkawa, T., Ami, Y., Suzaki, Y., Nagata, N., Sakata, H., Yamanouchi, K., and Kai, C. (1996). Nonhuman primate models of measles. *Lab. Anim. Sci.* 46, 315–320.

Lemon, K., de Vries, R. D., Mesman, A. W., McQuaid, S., van Amerongen, G., Yuksel, S., Ludlow, M., Rennick, L. J., Kuiken, T., Rima, B. K., Geijtenbeek, T. B. H., Osterhaus, A. D. M. E., Duprex, W. P., and de Swart, R. L. (2011). Early target cells of measles virus after aerosol infection of non-human primates. *PLoS Pathog.* 7, e1001263. doi:10.1371/journal.ppat.1001263

Leonard, V. H. J., Hodge, G., Valle, J. R., McChesney, M. B., and Cattaneo, R. (2010). Measles virus selectively blind to signaling lymphocytes activation molecule (SLAM; CD150) is attenuated and induces strong adaptive immune responses in rhesus monkeys. *J. Virol.* 84, 3413–3420.

Leonard, V. H. J., Sinn, P. L., Hodge, G., Miest, T., Devaux, P., Oezguen, N., Braun, W., McCray, P. B. Jr., McChesney, M. B., and Cattaneo, R. (2008). Measles virus blind to its epithelial cell receptor remains virulent in rhesus monkeys but cannot cross the airway epithelium and is not shed. *J. Clin. Invest.* 118, 2448–2458.

Levy, B. M., and Mirkovic, R. R. (1971). An epizootic of measles in a marmoset colony. *Lab. Anim. Sci.* 21, 33–39.

McChesney, M. B., Miller, C. J., Rota, P. A., Zhu, Y., Antipa, L., Lerche, N. W., Ahmed, R., and Bellini, W. J. (1997). Experimental measles. I. Pathogenesis in the normal and the immunized host. *Virology* 233, 74–84.

Muhlebach, M. D., Mateo, M., Sinn, P. L., Prufer, S., Uhlig, K. M., Leonard, V. H., Navaratnarajah, C. K., Frenzke, M., Wong, X. X., Sawatsky, B., Ramachandran, S., McCray, P. B. Jr., Cichutek, K., von Messling, V., Lopez, M., and Cattaneo, R. (2011). Adherens junction protein nectin-4 is the epithelial receptor for measles virus. *Nature* 480, 530–533.

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M., and Yanagi, Y. (2008). Measles virus circumvents the host interferon response by different actions of the C and V proteins. *J. Virol.* 82, 8296–8306.

Nakayama, T., Komase, K., Uzuka, R., Hoshi, A., and Okafuji, T. (2001). Leucine at position 278 of the AIK-C measles virus vaccine strain fusion protein is responsible for reduced syncytium formation. *J. Gen. Virol.* 82, 2143–2150.

Naniche, D., Varior-Krishnan, G., Cervoni, F., Wild, T. F., Rossi, B., Rabourdin-Combe, C., and Gerlier, D. (1993). Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J. Virol.* 67, 6025–6032.

Navaratnarajah, C. K., Leonard, V. H. J., and Cattaneo, R. (2009). Measles virus glycoprotein complex assembly, receptor attachment, and cell entry. *Curr. Top. Microbiol. Immunol.* 329, 59–76.

Nii, S., Kamahora, J., Mori, Y., Takahashi, M., Nishimura, S., and Okuno, Y. (1964). Experimental pathology of measles in monkeys. *Biken J.* 6, 271–297.

Noyce, R. S., Bondre, D. G., Ha, M. N., Lin, L. T., Sisson, G., Tsao, M. S., and Richardson, C. D. (2011). Tumor cell marker PVRL4 (nectin 4) is an epithelial cell receptor for measles virus. *PLoS Pathog.* 7, e1002240. doi:10.1371/journal.ppat.1002240

Peebles, T. C., McCarthy, K., Enders, J. F., and Holloway, A. (1957). Behavior of monkeys after inoculation of virus derived from patients with measles and propagated in tissue culture together with observations on spontaneous infections of these animals by an agent exhibiting similar antigenic properties. *J. Immunol.* 78, 63–74.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G., and Billeter, M. A. (1995). Rescue of measles virus from cloned DNA. *EMBO J.* 14, 5773–5784.

Russell, S. J., and Peng, K. W. (2009). Measles virus for cancer therapy. *Curr. Top. Microbiol. Immunol.* 330, 213–241.

Sakaguchi, M., Yoshikawa, Y., Yamanouchi, K., Sata, T., Nagashima, K., and Takeda, K. (1986). Growth of measles virus in epithelial and lymphoid tissues of cynomolgus monkeys. *Microbiol. Immunol.* 30, 1067–1073.

Sellin, C. I., and Horvat, B. (2009). Current animal models: transgenic animal models for study of measles pathogenesis. *Curr. Top. Microbiol. Immunol.* 330, 111–127.

Takeda, M., Ohno, S., Seki, F., Hashimoto, K., Miyajima, N., Takeuchi, K., and Yanagi, Y. (2005). Efficient rescue of measles virus from cloned cDNA using SLAM-expressing Chinese hamster ovary cells. *Virus Res.* 108, 161–165.

Takeda, M., Takeuchi, K., Miyajima, N., Kobune, F., Ami, Y., Nagata, N., Suzaki, Y., Nagai, Y., and Tashiro, M. (2000). Recovery of pathogenic measles virus from cloned cDNA. *J. Virol.* 74, 6643–6647.

Takeuchi, K., Miyajima, N., Kobune, F., and Tashiro, M. (2000). Comparative nucleotide sequence analyses of the entire genomes of B95a cell-isolated and Vero cell-isolated measles viruses from the same patient. *Virus Genes* 20, 253–257.

Takeuchi, K., Nagata, N., Kato, S., Ami, Y., Suzaki, Y., Suzuki, T., Sato, Y., Tsunetsugu-Yokota, Y., Mori, K., Nguyen, V. N., Kimura, H., and Nagata, K. (2012). Wild-type measles virus with the hemagglutinin protein of the Edmonston vaccine strain retains wild-type tropism in macaques. *J. Virol.* PMID: 22238320. [Epub ahead of print].

Takeuchi, K., Takeda, M., Miyajima, N., Ami, Y., Nagata, N., Suzaki, Y., Shahnewaz, J., Kadota, S., and Nagata, K. (2005). Stringent requirement for the C protein of wild-type measles virus for growth in vitro and in macaques. *J. Virol.* 79, 7838–7844.

Takeuchi, K., Takeda, M., Miyajima, N., Kobune, F., Tanabayashi, K., and Tashiro, M. (2002). Recombinant wild-type and Edmonston strain measles viruses bearing heterologous H proteins: role of H protein in cell fusion and host cell specificity. *J. Virol.* 76, 4891–4900.

Tatsuo, H., Ono, N., Tanaka, K., and Yanagi, Y. (2000). SLAM (CDw150) is a cellular receptor for measles virus. *Nature* 406, 893–897.

Terao-Muro, Y., Yoneda, M., Seki, T., Watanabe, A., Tsukiyama-Kohara, K., Fujita, K., and Kai, C. (2008). Heparin-like glycosaminoglycans prevent the infection of measles virus in SLAM-negative cell lines. *Antiviral Res.* 80, 370–376.

van Binnendijk, R. S., van der Heijden, R. W., van Amerongen, G., UytdeHaag, F. G., and Osterhaus, A. D. (1994). Viral replication and development of specific immunity in macaques after infection with different measles virus strains. *J. Infect. Dis.* 170, 443–448.

von Messling, V., Milosevic, D., and Cattaneo, R. (2004). Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14216–14221.

Yamanouchi, K., Egashira, Y., Uchida, N., Kodama, H., Kobune, F., Hayami, M., Fukuda, A., and Shishido, A. (1970). Giant cell formation in lymphoid tissue of monkeys inoculated with various strains of measles virus. *Jpn. J. Med. Sci. Biol.* 23, 131–145.

Zhu, Y., Heath, J., Collins, J., Greene, T., Antipa, L., Rota, P., Bellini, W., and McChesney, M. (1997). Experimental measles. II. Infection and immunity in the rhesus macaque. *Virology* 233, 85–92.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 November 2011; accepted: 09 January 2012; published online: 30 January 2012.

Citation: Kato S-i, Nagata K and Takeuchi K (2012) Cell tropism and pathogenesis of measles virus in monkeys. Front. Microbio. 3:14. doi: 10.3389/fmicb.2012.00014

This article was submitted to Frontiers in Virology, a specialty of Frontiers in Microbiology.

Journal of General Virology (2012), **93**, 1328–1338 DOI 10.1099/vir.0.036665-0

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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Received 21 July 2011
Accepted 12 February 2012

B23/nucleophosmin has been identified *in vitro* as a stimulatory factor for replication of adenovirus DNA complexed with viral basic core proteins. In the present study, the *in vivo* function of B23 in the adenovirus life cycle was studied. It was found that both the expression of a decoy mutant derived from adenovirus core protein V that tightly associates with B23 and small interfering RNA-mediated depletion of B23 impeded 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 increased 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, these results suggest that proper viral chromatin assembly, regulated in part by B23, is crucial for the maturation of infectious virus particles.

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).

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Supplementary methods and two supplementary figures are available with the online version of this paper.

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 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.*, 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 their interaction domains. Furthermore, we studied the effect of small interfering RNA (siRNA)-mediated knockdown of B23 on adenovirus proliferation. Perturbation of B23 function by overexpression of the decoy molecule or by knockdown was shown to impede adenovirus proliferation without significant inhibition of viral DNA replication or viral late gene expression. However, chromatin immunoprecipitation (ChIP) experiments indicated that the association of core proteins and cellular histones with viral DNA was significantly increased following B23 knockdown. Taken together, these 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 showed that B23 interacts with adenoviral core proteins V and VII (Samad *et al.*, 2007). However, the function of B23 in adenovirus proliferation has not yet been clarified. To gain additional insight into the *in vivo* function of B23, we designed a decoy molecule 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 overexpression 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

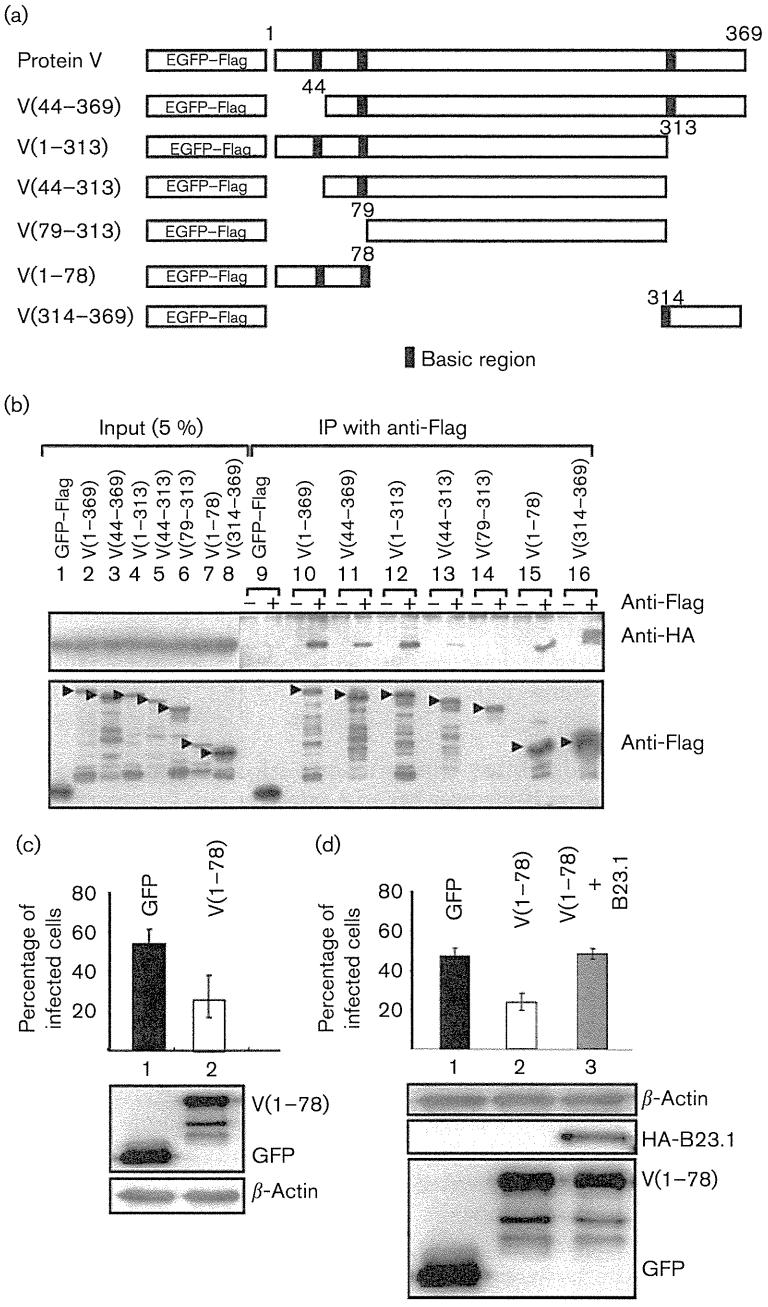

Fig. 1. N- and C-terminal regions of core protein V are required for the interaction with B23. (a) Schematic diagram of wild-type and mutant protein V constructs. GFP–Flag tag was fused at the N terminus of core protein V and its mutant proteins. The basic clusters of core protein V are indicated by filled boxes. (b) Immunoprecipitation of Flag-tagged core protein V. 293T cells were transiently co-transfected 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.

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

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 nucleolar 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 over-expression. 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 adenoviral 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).

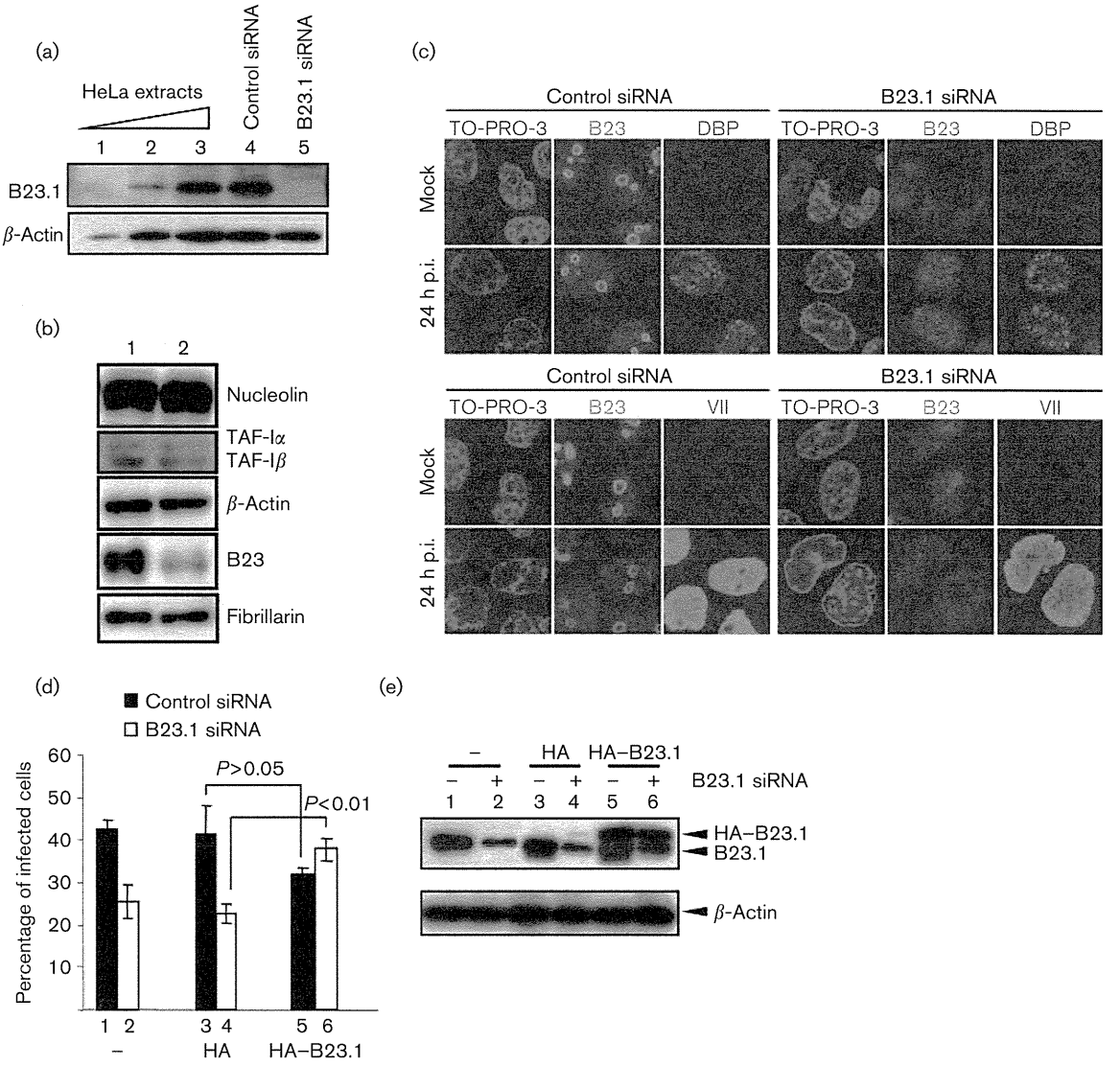

Fig. 2. B23 is involved in adenovirus proliferation. (a) Knockdown of B23.1. HeLa cells were transfected with control siRNA (lane 4) or B23 siRNA (lane 5), and the expression level of B23 was examined by Western blotting with anti-B23 antibody. HeLa cell lysates from 5×10^3, 1.5×10^4 and 5×10^4 cells (lanes 1–3, respectively) were loaded onto the same gel and used as standards. β-Actin is shown as a loading control. (b) Expression of nucleolar proteins. The expression levels of the indicated proteins were determined by Western blotting using HeLa cells treated with control siRNA (lane 1) or B23.1 siRNA (lane 2) 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, were superinfected with HAdV5 and subjected to indirect immunofluorescence analyses at 24 h p.i. B23 and DBP (top panels) or B23 and core protein VII (bottom panels) were simultaneously stained and visualized. Nuclei were counterstained with TO-PRO-3. (d) Inhibition of infectious virus production by knockdown of B23. HeLa cells treated with control or B23.1 siRNA were infected with HAdV5. At 24 h p.i., virus titres in the culture medium were determined as described in Methods (lanes 1 and 2). At 36 h after siRNA transfection, pCHA empty vector (lanes 3 and 4) or pCHA-B23.1 (lanes 5 and 6) was transfected into the cells and incubated for 24 h. The cells were then superinfected with HAdV5 and virus infectivity was determined at 24 h p.i., as described above. Experiments were carried out in triplicate and results are shown as means ± SD. Statistical *P* values are indicated at the top of the graph. (e) Expression level of endogenous and exogenous B23. Lysates prepared as described in (d) were analysed by Western blotting with anti-B23.1 and anti β-actin antibodies (top and bottom panels, respectively).

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

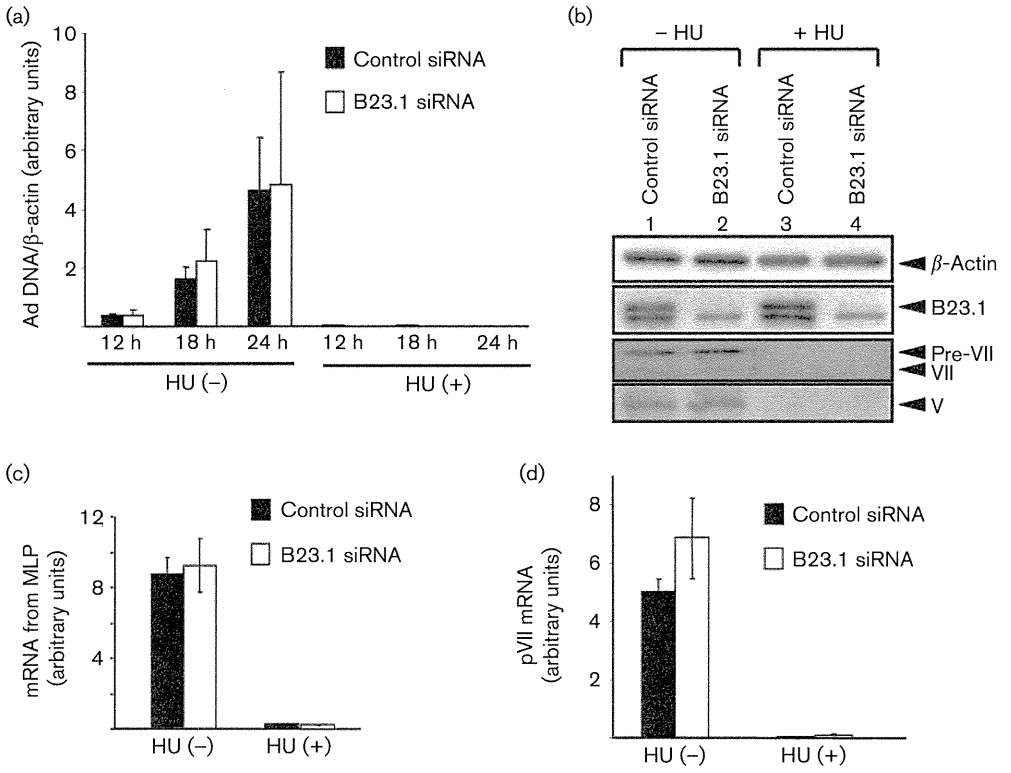

Fig. 3. Effect of B23 knockdown on adenoviral DNA replication and late gene expression. (a) Effect of B23.1 knockdown on adenoviral DNA replication. Control or B23.1 siRNA-treated HeLa cells were infected with HAdV5 and incubated without (–) or with (+) 2 mM hydroxyurea (HU). At 12, 18 and 24 h p.i., DNA was purified from the infected cells and the amount of viral DNA was examined by qPCR using a primer set specific for the VA region of the adenovirus genome. Genomic DNA purified from HeLa cells infected with HAdV5 was used as a standard for the amount of the virus genome in infected cells, and the relative amounts of adenoviral DNA were normalized to that of the β-actin gene. (b) Expression level of late gene products. Lysates were prepared from infected HeLa cells as described in (a) at 24 h p.i. Proteins were separated by SDS-PAGE, and analysed by Western blotting with antibodies against β-actin, B23 and core proteins V and pVII. (c, d) Expression levels of adenovirus late genes. HeLa cells treated with siRNA as described in (a) were infected with HAdV5. Total RNA was prepared at 18 h p.i., and the expression level of MLP mRNA (c) and pVII mRNA (d) was determined by qRT-PCR using specific primer sets, as described in Methods. PCRs were performed in triplicate and results are shown as means ± SD. Three independent experiments showed similar results.

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.*,

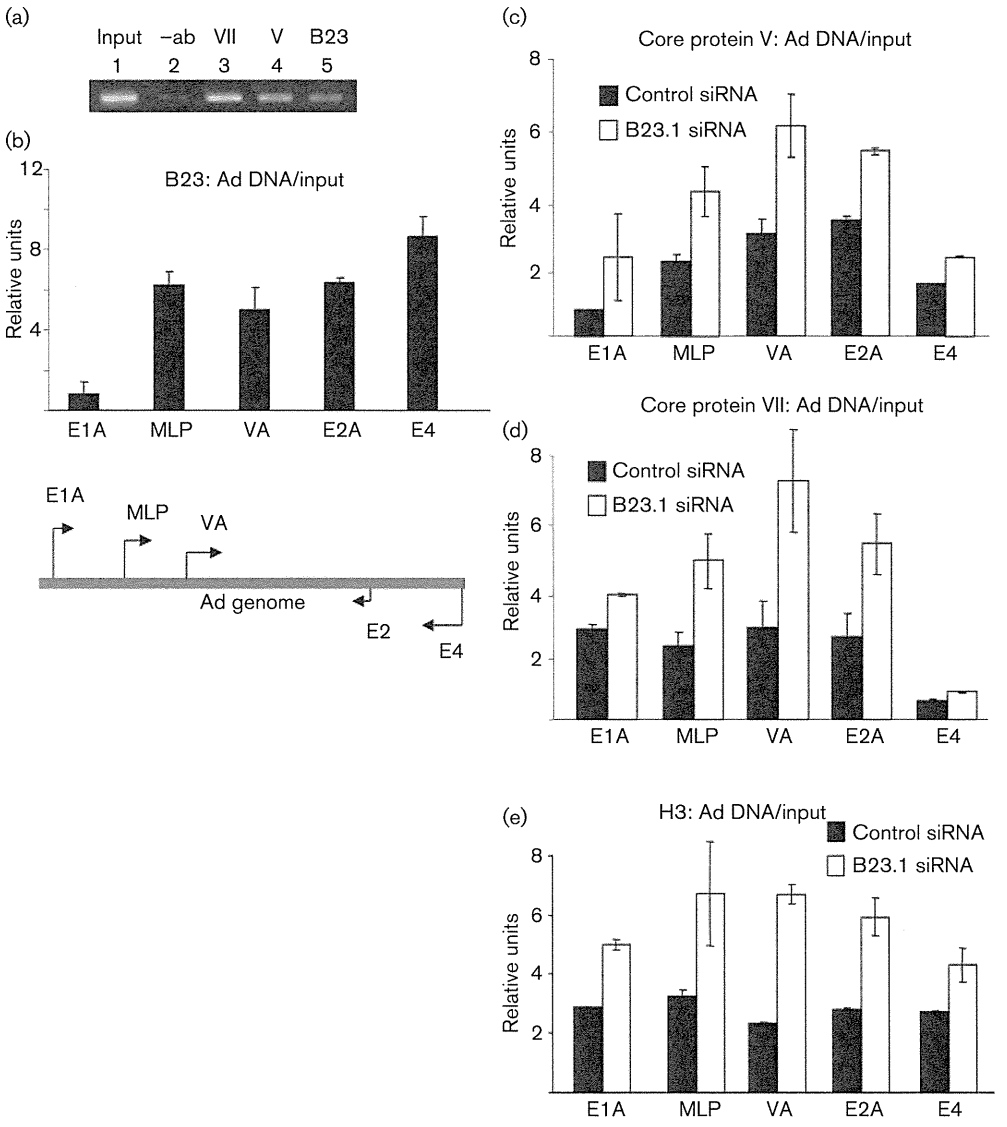

Fig. 4. Effect of B23 knockdown on adenovirus chromatin structure. (a) ChIP assays were carried out with extracts prepared from HAdV5-infected HeLa cells at 20 h p.i. DNA immunoprecipitated without (−ab, lane 2) or with anti-pVII, anti-V and anti-B23 antibodies (lanes 3–5, respectively) was examined by PCR using a primer set specific for the adenovirus VA gene. (b) B23 associates with the entire adenovirus genome in infected cells. DNA immunoprecipitated with anti-B23 as described in (a) was subjected to qPCR using primer sets specific for the adenovirus genome. The positions of the primer sets used are represented schematically in the lower panel. Arrows indicate the position and direction of transcription of each gene. DNA extracted from input extracts was used as a standard to quantify the amount of DNA immunoprecipitated with the anti-B23 antibody. (c–e) ChIP assay of adenovirus-infected HeLa cells treated with siRNAs. Control or B23.1 siRNA-treated HeLa cells were infected with HAdV5 and ChIP assays were carried out with antibodies against core proteins V (c) and pVII (d) and histone H3 (e). Immunoprecipitated DNA was examined by qPCR using the primer sets shown in (b). The amounts of immunoprecipitated DNA were analysed quantitatively and compared with those of DNAs extracted from input extracts. In (b–e), PCRs were carried out in triplicate and results are shown as means ± SD. Two independent experiments showed similar results.

1996; Matsumoto *et al.*, 1993; Okuwaki *et al.*, 2001a). 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

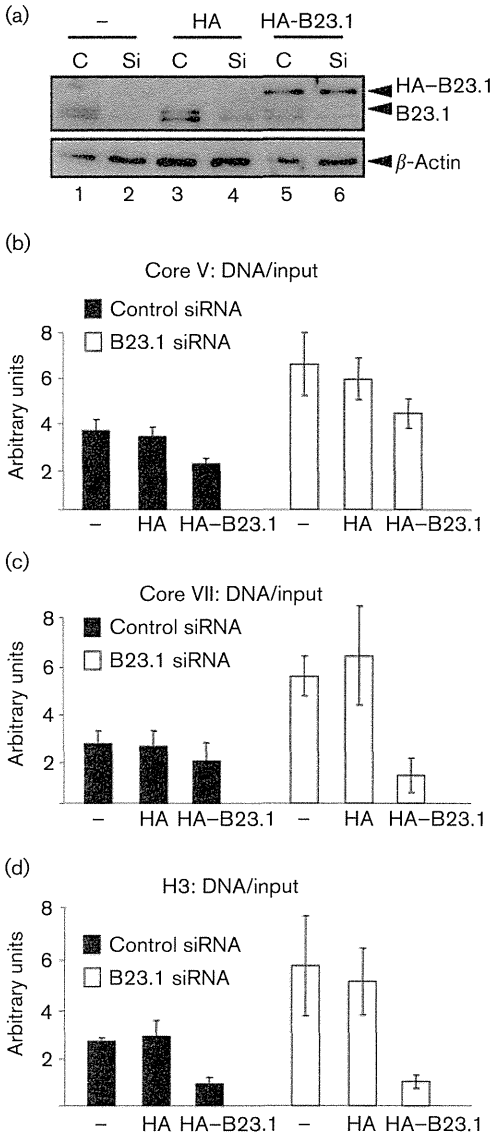

Fig. 5. Overexpression of exogenous B23.1 counteracts the effect of B23.1 knockdown on viral chromatin structure. (a) Expression levels of endogenous and exogenous B23.1. HeLa cells were treated with control (C) or B23.1 (Si) siRNA without (lanes 1 and 2) or with transfection of pCHA (lanes 3 and 4) 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 asmeans ± 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 B23ΔC2 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 20 h post-transfection, cells were superinfected with HAdV5 at an m.o.i. of 10. At 24 h p.i., 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 per well) to determine the virus titre. After incubation for 1 h, cells were supplemented with MEM containing 2 % FBS and maintained at 37 °C in a 5 % CO_2 environment for an additional 15 h. The cells on coverslips were collected, washed with PBS, fixed with 3 % paraformaldehyde for 10 min at room temperature and stained with anti-DBP antibody. Cells were counterstained with DAPI to stain the nuclei, and infectious titres (percentage of infected cells) were determined by counting DBP-positive cells compared with DAPI-positive cells. The assay was carried out in duplicate and the results are given as means $\pm$ SD.

B23 knock down by siRNA. Stealth RNAi negative control and B23 Stealth RNAi (NPM1-HSS143154; Invitrogen) were introduced into HeLa cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. At 24 h post-transfection, the medium was replaced and the cells were harvested at 60 h after siRNA transfection. Total HeLa cell lysates were prepared, and proteins were separated by SDS-PAGE (10 % acrylamide) and detected by Western blotting. To examine the effect of B23 knockdown on virus production, control and B23 siRNA-treated HeLa cells at 60 h post-transfection were infected with HAdV5 at an m.o.i. of 10. After 24 h, the culture medium was recovered and the virus titre determined, as described above.

Immunofluorescence assays. Indirect immunofluorescence assays were carried out essentially as described previously (Haruki *et al.*, 2006). Briefly, cells grown on 15 mm coverslips (Matsunami) were fixed with 4 % paraformaldehyde in PBS 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 He/Ne 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 (1×10^5) 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 (1×10^5) 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 mRNA from the MLP (5′-ACTCTCTTCCGCATCGCTGT-3′ and 5′-GTGACTG-GTTAGACGCCTTTCT-3′) and β-actin (5′-ATGGGTCAGAAGG-ATTCCTATGT-3′ and 5′-GGTCATCTTCTCGCGGTT-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′-GGGTCAAAGT-TGGCGTTTTA-3′ and 5′-CAAAATGGCTAGGAGGTGGA-3′ for the E1a promoter region, 5′-GCGGTCCTCCTCGTATAGAA-3′ and 5′-CCCACCCCCTTTTATAGCC-3′ for the MLP region, 5′-GCTG-GAGCAAAACCCAAATA-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′-GATTTTTACAATGGCCG-GACT-3′ for the E4 ORF region.

ACKNOWLEDGEMENTS

We thank Professor W. C. Russell for providing anti-V and anti-DBP antibodies. This work was supported in part by grants-in-aid from the Ministry of Culture, Sports, Science, and Technology of Japan (to K. N. and M. O.), Special Coordination Funds for Promoting Science and Technology (to M. O.), and a research fellowship of the Japan Society for the Promotion of Science (JSPS) (to M. A. S.).

REFERENCES

Adachi, Y., Copeland, T. D., Hatanaka, M. & Oroszlan, S. (1993). Nucleolar targeting signal of Rex protein of human T-cell leukemia virus type I specifically binds to nucleolar shuttle protein B-23. *J Biol Chem* **268**, 13930–13934.

Anderson, C. W., Young, M. E. & Flint, S. J. (1989). Characterization of the adenovirus 2 virion protein, mu. *Virology* **172**, 506–512.

Black, B. C. & Center, M. S. (1979). DNA-binding properties of the major core protein of adenovirus 2. *Nucleic Acids Res* **6**, 2339–2353.

Brown, D. T., Westphal, M., Burlingham, B. T., Winterhoff, U. & Doerfler, W. (1975). Structure and composition of the adenovirus type 2 core. *J Virol* **16**, 366–387.

Chatterjee, P. K., Vayda, M. E. & Flint, S. J. (1985). Interactions among the three adenovirus core proteins. *J Virol* **55**, 379–386.

Chatterjee, P. K., Vayda, M. E. & Flint, S. J. (1986). Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. *EMBO J* **5**, 1633–1644.