Minute Virus of Mice Inhibits Transcription of the Cyclin B1 Gene during Infection

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ABSTRACT Replication of minute virus of mice (MVM) induces a sustained cellular DNA damage response (DDR) which the virus then exploits to prepare the nuclear environment for effective parvovirus takeover. An essential aspect of the MVM-induced DDR is the establishment of a potent premitotic block, which we previously found to be independent of activated p21 and ATR/Chk1 signaling. This arrest, unlike others reported previously, depends upon a significant, specific depletion of cyclin B1 and its encoding RNA, which precludes cyclin B1/CDK1 complex function, thus preventing mitotic entry. We show here that while the stability of cyclin B1 RNA was not affected by MVM infection, the production of nascent cyclin B1 RNA was substantially diminished at late times postinfection. Ectopic expression of NS1 alone did not reduce cyclin B1 expression. MVM infection also reduced the levels of cyclin B1 protein, and RNA levels normally increased in response to DNA-damaging reagents. We demonstrated that at times of reduced cyclin B1 expression during infection, there was a significantly reduced occupancy of RNA polymerase II and the essential mitotic transcription factor FoxM1 on the cyclin B1 gene promoter. Additionally, while total FoxM1 levels remained constant, there was a significant decrease of the phosphorylated, likely active, forms of FoxM1. Targeting of a constitutively active FoxM1 construct or the activation domain of FoxM1 to the cyclin B1 gene promoter via clustered regularly interspaced short palindromic repeats (CRISPR)-enzymatically inactive Cas9 in MVM-infected cells increased both cyclin B1 protein and RNA levels, implicating FoxM1 as a critical target for cyclin B1 inhibition during MVM infection.

IMPORTANCE Replication of the parvovirus minute virus of mice (MVM) induces a sustained cellular DNA damage response (DDR) which the virus exploits to prepare the nuclear environment for effective takeover. An essential aspect of the MVM-induced DDR is establishment of a potent premitotic block. This block depends upon a significant, specific depletion of cyclin B1 and its encoding RNA that precludes cyclin B1/CDK1 complex functions necessary for mitotic entry. We show that reduced cyclin B1 expression is controlled primarily at the level of transcription initiation. Additionally, the essential mitotic transcription factor FoxM1 and RNA polymerase II were found to occupy the cyclin B1 gene promoter at reduced levels during infection. Recruiting a constitutively active FoxM1 construct or the activation domain of FoxM1 to the cyclin B1 gene promoter via CRISPR-catalytically inactive Cas9 (dCas9) in MVM-infected cells increased expression of both cyclin B1 protein and RNA, implicating FoxM1 as a critical target mediating MVM-induced cyclin B1 inhibition.

KEYWORDS DNA damage checkpoints, minute virus of mice, parvovirus, virology

Minute virus of mice (MVM) is an autonomously replicating parvovirus which induces a robust ATM-dependent DNA damage response (DDR) (1). Inhibition of this response significantly reduces viral replication (1). The virally induced DDR results

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in the phosphorylation of several proteins important for DNA repair, including H2AX, p53, Chk2, NBS1, and RPA32. Numerous DDR proteins colocalize within viral replication centers, termed autonomous parvovirus-associated replication (APAR) bodies, suggesting that MVM may recruit many of these proteins either for direct usage or to sequester them. Taken together, our observations suggest that the DDR plays an important role during MVM replication.

MVM infection results in an extended premitotic cell cycle block during which viral replication proceeds. We previously showed that this cell cycle block correlates with the activation of Chk2 and the loss of CDC25A phosphatase activity (2). Experiments designed to monitor cell cycle progression following infection revealed that the G₂/M block imposed by MVM prevents entry of infected cells into mitosis as measured by the lack of Ser-28 phosphorylation of histone H3 (2). Normally, entry into mitosis is tightly regulated and requires the cyclin B1/CDK1 complex to reach threshold levels of activity (3). Importantly, the kinase activity of the cyclin B1/CDK1 complex in MVM-infected cells was dramatically reduced compared to the activity of this complex in cells that were chemically trapped in early mitosis by use of nocodazole. These observations thus suggested that low cyclin B1/CDK1 activity is an important factor in maintaining the premitotic cell cycle block during MVM infection. Surprisingly, we found that although the inhibitory phosphorylation of CDK1 at Tyr-15 was lost during infection—an indicator typically associated with activity of the cyclin B1/CDK1 complex—cyclin B1 and its encoding RNA were substantially depleted (2). These results suggested that MVM was regulating the stability or production of cyclin B1 RNA to prevent cyclin B1/CDK1 activity, thus affecting this critical premitotic checkpoint.

The nine-intron-containing cyclin B1 gene (*Ccnb1*) is regulated in a complex manner during cell cycle progression (3, 4). It has at least five transcriptional promoter elements that regulate its expression. These include CCAAT boxes, GC boxes, E boxes, p53-responsive elements, and the cell cycle gene homology region (CHR), which bind NF-Y, SP1, Myc, p53, and the Myb-MuvB (MMB) complex, respectively (5–13). The CHR and its protein binding partners are important contributors to the intricate regulation of the cyclin B1 gene (14). Many cell cycle genes in addition to the cyclin B1 gene are regulated via CHR sites, including the cyclin B2, CDK1, CDC25, and Polo-like kinase 1 (PLK1) genes (15). CHR-regulated genes are typically active during G₂/M and repressed during other stages of the cell cycle. The DREAM complex both represses the transcriptional activity of these CHR genes during G₀ and G₁ and helps to activate them as cells transit from late G₁ into S phase and progress toward the mitotic border (16). As cells progress through late S and into G₂/M, the DREAM complex dissociates from the cellular promoter, and MuvB remains and successively recruits both B-Myb, forming the MMB complex, and the forkhead transcription factor FoxM1 (17). Phosphorylation of five to seven sites that reside within the C-terminal transactivation domain is critical for FoxM1 function (18). These phosphorylation events, some of which depend upon the kinase activity of the cyclin A/CDK complex, are thought to activate FoxM1 by relieving autorepression caused by the interaction of the C terminus with an N-terminal repressive domain (19). The expression of the cyclin B1 gene can also be regulated at the level of its RNA stability (20). Interactions of the cellular RNA binding protein HuR within the 3' untranslated region (UTR) have been shown to affect the stability of cyclin B1 RNA (21, 22).

In this study, we characterized how MVM infection reduces expression of the RNA encoding cyclin B1. MVM infection resulted in reduced levels of nascent cyclin B1 RNA, which in turn were associated with a significantly reduced occupancy of RNA polymerase II (RNA Pol II) on the cyclin B1 gene promoter. Binding of the transcription factor FoxM1 to the cyclin B1 gene promoter was also significantly reduced during infection, as was detection via Western blotting of the phosphorylated, likely active, forms of FoxM1. Gain-of-function experiments in which a constitutively active FoxM1 construct or the FoxM1 transactivation domain was targeted to the cyclin B1 gene promoter, using dead-Cas9 (dCas9)–FoxM1 fusion proteins in the presence of cyclin B1 gene promoter guide RNAs, increased both cyclin B1 RNA and protein expression levels in

FIG 1 Cyclin B1 protein and RNA are reduced during MVM infection. (A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. In a separate experiment, at 15 h postrelease, cells were treated with 200 nM doxorubicin (Doxo) as indicated. Cells were harvested at the indicated time points (Tx, with x given in hours), and RIPA lysates were Western blotted using antibodies directed against the indicated proteins. Entry into S phase occurs approximately 12 h after release into complete medium. Virally infected cells harvested at 24 h thus represent an approximately 12-h transit into S phase. (B) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. Cells were harvested at the indicated time points, and total RNA was isolated using TRIzol reagent. Samples were processed for RNase protection assay (RPA) with a probe against cyclin B1 or actin as described in Materials and Methods. (C) Human NB324K cells were parasynchronized by isoleucine deprivation and infected with MVM or MVM-1989 at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. Cells were harvested at the indicated time points, and RIPA lysates were Western blotted using antibodies directed against the indicated proteins. (D) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. Cells were harvested at 30 h postrelease, and RIPA lysates were Western blotted using antibodies directed against the indicated proteins. (E) Murine A9 cells were treated as described for panel D. Cells were harvested at 30 h postrelease, and total RNA was isolated using TRIzol reagent. Samples were processed for RPA with a probe against cyclin B1 or GAPDH. (F) Murine A9 cells were parasynchronized by isoleucine deprivation and either infected (MVM) or not (mock) with MVM at an MOI of 10 at the time of release into complete medium. Alternatively, at 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. At 24 h postinfection, cells were then processed for flow cytometry as described in Materials and Methods. Data were processed in FlowJo (FlowJo, LLC) and are presented as percentages of the total per cell cycle phase.

infected cells. These results implicated FoxM1 as a critical target for cyclin B1 inhibition by MVM which contributes to the premitotic block evoked during viral infection.

RESULTS

Cyclin B1 protein and RNA levels are reduced during MVM infection. At late times during MVM infection, when cells are arrested prior to mitosis, levels of cyclin B1 and its encoding RNA are reduced (2). In our study, following a modest transient increase, depletion of cyclin B1 in MVM-infected murine A9 cells could again be seen (Fig. 1A) (at least a 20-fold decrease at 32 h [T32] [compare lane 6 to lane 5]; cells were parasynchronized by isoleucine deprivation and infected as described in Materials and Methods). Treatment with the topoisomerase II inhibitor doxorubicin (23) blocks cells prior to mitosis, similarly to MVM infection; however, in contrast to MVM infection, treatment with doxorubicin induced high levels of cyclin B1 (at least 2-fold over mock

treatment levels) (Fig. 1A, lanes 7 to 9). This result suggested that the depletion of cyclin B1 was MVM specific, not merely a general characteristic of cells prevented from mitotic entry. Transient expression of MVM NS1 alone did not reduce cyclin B1 expression (data not shown). Typical cell cycle profiles of MVM-infected and doxorubicin-treated para-synchronized A9 cells are shown in Fig. 1F.

Cyclin B1 is typically degraded as cells cycle from mitosis into G₁. However, both proteasome and protease inhibitors had only a negligible ability to restore cyclin B1 levels during infection (data not shown). Additionally, efficient small interfering RNA (siRNA) knockdown (done as previously reported [24]) of the CDC20 E3 ubiquitin ligase, which targets cyclin B1 for ubiquitination during G₁, also had no effect on the accumulation of cyclin B1 in MVM-infected cells (data not shown). However, analysis of RNAs from the 18-h and 24-h samples from the experiment shown in Fig. 1A demonstrated that cyclin B1 RNA was significantly depleted (approximately 6- to 7-fold) by this time point (Fig. 1B, lane 4).

A similar depletion of cyclin B1 could be seen following MVM infection of human NB324K cells, at both the protein level (Fig. 1C, lanes 3, 7, and 11) and the RNA level (data not shown), although the timing seemed to be accelerated compared to that in A9 cells. MVM mutants that lack the viral NS2 protein can replicate in NB324K cells, and infection with such a mutant (NS2-1989) also resulted in the loss of cyclin B1, indicating that NS2 is not involved in this loss during infection of these cells (Fig. 1C, lanes 4, 8, and 12).

Interestingly, MVM infection greatly reduced the high levels of cyclin B1 protein (at least 20-fold) and RNA (at least 5-fold) normally induced by doxorubicin treatment (Fig. 1D, compare lane 3 to lane 1, and E, compare lane 6 to lane 4). These results suggested that MVM can play a dominant role in the effects of a strong exogenous DNA-damaging agent, consistent with our conclusion that MVM's role is specific.

Nascent cyclin B1 RNA production is reduced during infection. Cyclin B1 mRNA levels are known to vary across the cell cycle. In addition to being subjected to degradation as discussed above, in normally cycling cells cyclin B1 expression is also controlled at the levels of transcription initiation and RNA stability (3, 6, 7, 20, 22). Extensive characterization of cyclin B1 RNA during MVM infection, when cyclin B1 protein levels were reduced, revealed no significant changes to the stability of cyclin B1 RNA (data not shown). We next chose to determine whether the cyclin B1 gene promoter or 3' end (UTR and polyadenylation site) might confer responsiveness to MVM infection. For this analysis, we examined the effect of MVM infection on a cyclin B1 cDNA representing only its open reading frame (ORF) (from AUG to TAA) driven by the heterologous tet-responsive element-inducible promoter (TRE2) and utilizing a simian virus 40 (SV40) polyadenylation signal, expressed from an integrated lentivirus vector (3XFLAG-Cyclin B1 [described in Materials and Methods]). The cloned cyclin B1 ORF was tagged with a triplet FLAG sequence to allow detection of the ectopically expressed protein. In addition, we developed an RNase protection probe across the tagged region to separately detect both the endogenous and ectopically expressed cyclin B1 RNAs by using a single probe within the same sample. As shown in Fig. 2A, while the endogenous cyclin B1 RNA was significantly reduced by MVM at 32 h postinfection, expression of cyclin B1 RNA from the ectopically expressed construct was reduced only slightly (Fig. 2A, compare lanes 5 and 6 to lanes 7 and 8). These findings were recapitulated at the level of protein accumulation (Fig. 2B, compare lane 3 to lane 2; in this figure, the ectopically expressed protein represents only a fraction of the total cyclin B1—its triple tag was readily detected by the anti-FLAG antibody, but it was below the level of detection by an antibody to cyclin B1 itself). These results confirmed that the primary effect of MVM on cyclin B1 expression was at the RNA rather than the protein level and suggested that it was mediated by either the cyclin B1 gene promoter, 3' UTR, or polyadenylation site.

We suspected that MVM might affect transcriptional initiation of the cyclin B1 gene, so we compared the expression levels of nascent pre-mRNA from the endogenous

FIG 2 Cyclin B1 driven from a heterologous promoter and 3' UTR is stable during MVM infection, but nascent cyclin B1 RNA production is reduced during infection. (A) Murine A9 cell lines inducibly expressing 3XF-cyclin B1 were generated as described in Materials and Methods. Cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. Cells were induced with doxycycline, as indicated, at the time of release. Cells were harvested at the indicated time points, and total RNA was isolated using TRIzol reagent. Samples were processed for RPA with a probe against 3XF-cyclin B1 (which separately detects FLAG-tagged and endogenous cyclin B1 RNAs) or actin. (B) The inducible murine A9 cell lines described for panel A were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. Cells were induced with doxycycline, as indicated, at the time of release. Cells were harvested at 34 h postinfection, and RIPA lysates were Western blotted using antibodies directed against the indicated proteins. (C) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. DRB was added at 40 μ g/ml at 24 h postinfection and incubated for 3 h. Cells were then washed with PBS and incubated in complete medium for the indicated time points. Total RNA was isolated using TRIzol, and samples were assayed by reverse transcription-qPCR as described in Materials and Methods. Data are presented as relative expression changes for three independent experiments. 18S rRNA was used for normalization of signals.

cyclin B1 gene in uninfected and infected cells. As shown in Fig. 2C, within 3 min following resumption of transcription after pausing induced by 5,6-dichlorobenzimidazole 1- β -D-ribofuranoside (DRB) treatment, MVM infection significantly reduced RNA elongation into the 5' end of exon 2 of cyclin B1, approximately 700 nucleotides downstream of the transcription start site, relative to that in mock-infected cells. As expected, doxorubicin-treated cells showed a substantial increase in nascent cyclin B1 RNA generation over that in mock-treated cells. The decreased nascent transcript accumulation in MVM-infected cells remained considerably depressed over the 10-min time course of the experiment. These quantitative real-time PCR results suggest a decreased rate of transcription compared to that in cycling mock-infected cells, with an even greater difference in rates relative to those in doxorubicin-treated cells blocked at a stage of the cell cycle similar to that for infected cells. These findings focused further attention on the initiation of transcription of the cyclin B1 gene as a target for MVM during infection.

MVM infection reduces occupancy of RNA polymerase II on the cyclin B1 gene promoter in a chromatin-independent manner. Repression of cell cycle gene ex-

FIG 3 MVM infection reduces occupancy of RNA polymerase II on the cyclin B1 gene (*Ccnb1*) promoter, although the local chromatin architecture is consistent with an open configuration. (A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. At 24 h postinfection, cells were processed as described in Materials and Methods for chromatin immunoprecipitation (ChIP) using antibodies directed against a histone modification associated with transcriptionally repressed chromatin, i.e., H3K9me3. Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as mean values for percentages of the input signal for at least three independent experiments (with standard errors of the means [SEM]). Note the difference in scale of the x axes for panels A and B. (B) Murine A9 cells were treated and analyzed as described for panel A, using antibodies directed against a histone modification associated with transcriptionally active chromatin, i.e., H3K4me2. Data are presented as mean values for percentages of the input signal for at least three independent experiments (with SEM). (C) Murine A9 cells were treated and analyzed as described for panel A, using antibodies directed against the paused, promoter-associated (CTD S5), phosphorylated RNA Pol II. Data are presented as mean values for percentages of the input signal for at least three independent experiments (with SEM). ****, $P \leq 0.0001$ (t test); ns, no significant difference. Note the difference in scale of the x axes for panels C and D. (D) Murine A9 cells were treated and analyzed as described for panel A, using antibodies directed against the elongating (CTD S2), phosphorylated RNA Pol II. Data are presented as mean values for percentages of the input signal for at least three independent experiments (with SEM). **, $P \leq 0.01$ (t test); ***, $P \leq 0.001$ (t test); ns, no significant difference. AU, arbitrary units.

pression may be caused by changes in local chromatin modifications and accessibility (25). However, the chromatin profile of the cyclin B1 gene promoter region during infection did not show a correlation with selected repressive chromatin marks. As shown in Fig. 3A, histone H3 trimethylated at lysine 9 (H3K9me3), which is associated with transcriptionally repressive chromatin, was found at similarly low levels at the transcription start site of the cyclin B1 gene in MVM-infected, doxorubicin-treated, and mock-treated cells. Reciprocally, histone H3 dimethylated at lysine 4 (H3K4me2), a histone modification associated with active chromatin, had a similar profile over the same region in infected, doxorubicin-treated, and mock-treated cells (Fig. 2B). Taken together, these results suggest that cyclin B1 gene transcription is not substantially altered by perturbations in the accessibility of local chromatin.

Despite the transcriptionally permissive modification of the chromatin associated with the cyclin B1 gene promoter during MVM infection, we detected a significant

difference in RNA Pol II occupancy in infected cells compared to that in either mock- or doxorubicin-treated cells. Chromatin immunoprecipitation (ChIP) assays using antibodies to either the promoter-associated (paused) form of RNA Pol II phosphorylated on the carboxy-terminal domain (CTD) at serine 5 (S5) (Fig. 3C) or the elongating, CTD-S2-phosphorylated RNA Pol II (S2) (Fig. 3D) showed significant reductions in occupancy during infection. This suggested that the decrease in nascent RNA seen in Fig. 2 was caused by a failure of RNA Pol II to engage the cyclin B1 gene promoter. In preliminary experiments that expanded upon this observation, we found that during infection, the expression of numerous cellular genes was reduced, while for many genes, expression was increased or unchanged (data not shown). These findings will be the focus of future studies; however, they suggest that reduction of cyclin B1 RNA was not merely the result of a nonspecific inhibition of all cellular RNAs during infection.

The transcription factor FoxM1 exhibits reduced occupancy on the cyclin B1 gene promoter during MVM infection. Expression of the cyclin B1 gene is governed by multiple transcription factors, including the heterotrimer NF-Y, SP1, Myc, p53, the MMB complex, and the DREAM complex (5–13). As described above, CHR-regulated genes are typically active during G₂/M and are repressed during other stages of the cell cycle. The DREAM complex both represses the transcriptional activity of these CHR genes during G₀ and G₁ and helps to activate them as cells transit from late G₁ into S phase and progress toward the mitotic border. As cells transition from G₁ to S phase, the repressive DREAM complex is dissociated, leaving MuvB bound to the promoter at CHR sites, which then successively recruits B-Myb, forming the MMB complex, and the transcription factor FoxM1 (17). FoxM1 must be activated via hyperphosphorylation, mostly concentrated in its transactivation domain, to stimulate transcription of the cyclin B1 gene by RNA Pol II (17–19, 26–28).

Although p53 can regulate cyclin B1 expression (29) and p53 is activated during MVM infection (1), efficient knockdown of p53 in infected cells had no detectable effect on cyclin B1 expression (data not shown). And while transcriptional activation of CHR-regulated genes has been shown to require binding of the heterotrimer NF-Y transcription factor (5, 14, 15), there was no significant difference in NF-YA occupancy at the cyclin B1 gene promoter in MVM-infected cells compared to that in doxorubicin-treated or mock-treated cells (Fig. 4A). Similarly, there was no significant difference in B-Myb occupancy in MVM-infected cells compared to that in mock-treated controls, and there was only a minor difference compared to that in doxorubicin-treated cells (Fig. 4B). Strikingly, however, FoxM1 deposition at the cyclin B1 gene promoter was reduced in MVM-infected cells compared to that in mock-treated cells and was substantially diminished relative to that in doxorubicin-treated cells residing at a similar stage of the cell cycle (Fig. 4C). Furthermore, the occupancy of FoxM1 at the cyclin B1 gene promoter reduced progressively as infection proceeded (Fig. 4D). These findings suggested that a reduction in FoxM1 engagement with the cyclin B1 gene promoter may have mediated MVM repression of cyclin B1 gene expression.

MVM infection reduces levels of phosphorylated forms of FoxM1. Targeting of the FoxM1 transactivation domain to the cyclin B1 gene promoter partially restored cyclin B1 expression. The transcriptional activity of FoxM1 depends upon phosphorylation of at least five to seven key residues within the C-terminal transactivation domain (18). Although the total levels of FoxM1 were not significantly altered during infection (Fig. 5A), we observed significant reductions in the levels of phosphorylated forms of FoxM1 (PP-FoxM1) in MVM-infected cells at 25 and 30 h postinfection compared to those in mock-infected or doxorubicin-treated controls (Fig. 5A, compare lanes 4 to 6 and lanes 7 to 9). These results suggested that the effect of MVM on cyclin B1 expression may have been due to a reduction of the active forms of FoxM1 identified by others (18, 19, 27).

Activation of FoxM1 requires phosphorylation by the cyclin A/CDK complex, which relieves autorepression conferred via its N-terminal domain (19). Transient transfection of a constitutively active FoxM1 mutant lacking its autoinhibitory N-terminal region

FIG 4 The transcription factor FoxM1 exhibits reduced occupancy on the cyclin B1 gene promoter during MVM infection. (A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. At 24 h postinfection, cells were processed as described in Materials and Methods for ChIP using antibodies directed against NF-YA. Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as mean values for percentages of the input signal for at least three independent experiments (with SEM). ns, no significant difference. (B) Murine A9 cells were treated and analyzed as described for panel A, using antibodies directed against B-Myb. *, $P \leq 0.05$ (t test); ns, no significant difference. (C) Murine A9 cells were treated and analyzed as described for panel A, using antibodies directed against FoxM1. **, $P \leq 0.01$ (t test); ***, $P \leq 0.001$ (t test); ****, $P \leq 0.0001$ (t test). Note the difference in scale of the x axes of panel C and panels A and B. (D) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. At the indicated time points (18, 24, and 32 h postinfection), cells were processed as described in Materials and Methods for ChIP using antibodies directed against FoxM1. Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as mean relative occupancies, as previously described (17), calculated from three experimental data points ($\pm$ SEM).

(Δ N-FoxM1) (19) failed to enhance cyclin B1 expression during MVM infection, even though this mutant retained its DNA binding domain (data not shown). However, when the mutant protein was fused to the C terminus of a catalytically inactive Cas9 protein (dCas9) and targeted to the cyclin B1 gene promoter by cyclin B1-specific, but not scrambled, guide RNAs, transient expression of the Δ N-FoxM1 mutant protein (dCas9- Δ N-FoxM1) led to substantial enhancements of both cyclin B1 protein and RNA (Fig. 5B and C, respectively) at 30 h post-MVM infection, when expression of cyclin B1, monitored in parallel, was reduced (data not shown).

To confirm these results, we also fused a FoxM1 transactivation domain construct in which phosphomimetic aspartic acids had been replaced with residues essential for activation (18) to the catalytically inactive Cas9 nuclease (dCas9-FoxM1-phospho-TAD). This construct does not contain the endogenous FoxM1 DNA binding domain. Similar to the results for the constitutively active dCas9- Δ N-FoxM1 fusion described above,

FIG 5 Levels of phosphorylated FoxM1 are reduced during MVM infection. Targeting of a constitutively active FoxM1 construct or a phosphomimetic transactivation domain of FoxM1 to the cyclin B1 gene promoter partially restores cyclin B1 expression. (A) Murine A9 cells were parasynchronized by isoleucine deprivation and infected with MVM at an MOI of 10 at the time of release into complete medium. At 15 h postrelease, cells were treated with 200 nM doxorubicin as indicated. Cells were harvested at the indicated time points, and RIPA lysates were Western blotted using antibodies directed against the indicated proteins. (B) Murine A9 cells were transfected during parasynchronization by isoleucine deprivation with plasmids containing no guide RNAs (gRNAs) or cyclin B1 gene promoter-targeting guide RNAs, catalytically inactive Cas9 fused to constitutively active FoxM1 (Δ N-FoxM1), and human CD4, as indicated. Cells were released into complete medium and infected with MVM at an MOI of 10. At 30 h postinfection, transfected cells were positively selected by CD4 expression as described in Materials and Methods. Whole-cell lysates were assayed by Western blotting using antibodies directed against the indicated proteins. (C) Total RNA was isolated with TRIzol reagent from the murine A9 cells described for panel B. Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as fold changes normalized to GAPDH and calculated by the $\Delta\Delta C_T$ method. (D) Murine A9 cells were treated as described for panel B with catalytically inactive Cas9 fused to a FoxM1 phosphomimetic transactivation domain and human CD4 as indicated. Cells were released into complete medium and infected with MVM at an MOI of 10. At 30 h postinfection, transfected cells were positively selected by CD4 expression as described in Materials and Methods. Whole-cell lysates were assayed by Western blotting using antibodies directed against the indicated proteins. (E) Total RNA was isolated with TRIzol reagent from the murine A9 cells described for panel D. Samples were analyzed by qPCR as described in Materials and Methods. Data are presented as fold changes normalized to GAPDH and calculated by the $\Delta\Delta C_T$ method.

when dCas9-FoxM1-phospho-TAD was targeted to the cyclin B1 gene promoter by cyclin B1-specific, but not scrambled, guide RNAs, transient expression of dCas9-FoxM1-phospho-TAD led to substantial enhancements of both cyclin B1 protein (Fig. 5D) and mRNA (Fig. 5E) levels at 30 h post-MVM infection. These results suggest that FoxM1 is a critical target for cyclin B1 inhibition by MVM.

DISCUSSION

Parvoviruses induce a potent premitotic cell cycle block during which replication continues for an extended period. MVM utilizes an unusual strategy to impose this

restriction, one which involves inhibiting transcription of the cyclin B1 gene. A key feature of this block is the MVM-induced depletion of phosphorylated forms of FoxM1, a key transcription factor regulated by the DREAM/MMB complexes. Targeting the transactivation domain of FoxM1 to the cyclin B1 gene promoter during MVM infection was able to restore significant expression of cyclin B1, implicating FoxM1 as being directly involved in MVM-dependent depletion of cyclin B1 expression.

Our initial investigations showed that the proteasome inhibitor MG132 could recover cyclin B1 levels during MVM infection to only a small degree—no greater than would be expected for cells that had evaded our blocking procedure (data not shown). Together with the observation that levels of cyclin B1 mRNA were reduced in infection, these results focused our attention on mechanisms that might account for depletion of cyclin B1 mRNA. In our first experiments, we monitored the effect of MVM infection on transiently transfected reporter genes engineered to either be driven by heterologous promoters or contain heterologous 3' ends. These experiments gave equivocal results, perhaps due to the overwhelming nature of the transfected reporter, as well as discrepancies between transfection and infection efficiencies. However, examination of the cyclin B1 RNAs expressed from integrated lentiviral vectors suggested that differences in cyclin B1 RNA levels during infection were modulated by either the 5' or 3' end of the gene. That these results were also reflected in the levels of cyclin B1 protein supported the notion that protein stability was not the primary cause of cyclin B1 depletion. We detected little difference in the stability of cyclin B1 RNA in infected cells; however, examining the production of nascent RNA directly from the endogenous cyclin B1 gene during infection suggested that MVM inhibited the production of cyclin B1 RNA. These results were supported by ChIP assays that demonstrated a significant reduction in RNA Pol II occupancy during MVM infection. However, these findings did not preclude possible additional effects attributable to the cyclin B1 3' UTR at specific points during infection. Initiation of transcription of the cyclin B gene is complex, and the DREAM complex has been shown to be critical for its cell cycle regulation. MVM induces the depletion of phosphorylated forms of FoxM1, a critical transcription factor regulated by the DREAM/MMB complexes, and we detected less occupancy of both RNA Pol II and FoxM1 at the cyclin B1 gene promoter during infection. Targeting of a constitutively active FoxM1 construct or the transactivation domain of FoxM1 to the cyclin B1 gene promoter during MVM infection regained a significant amount of cyclin B1 expression, supporting a role for FoxM1 as a target of the regulation of cyclin B1 by MVM.

Importantly, the reduction of cyclin B1 RNA during infection is likely not simply the result of a global repression of cellular transcription. We recently began an investigation of the expression levels of multiple cellular genes during infection. The preliminary results are complex but identify genes whose expression is reduced, increased, or not significantly changed. These findings will be the focus of future studies; however, they suggest that the reduction of cyclin B1 RNA is not merely the result of a nonspecific inhibition of all cellular RNAs during infection.

How MVM reduces phosphorylation of FoxM1 is not yet clear. Cyclin A/CDK activity is required for alleviation of FoxM1 autorepression, and Chk2 activity is important for regulating FoxM1 levels during a DDR (19, 30). Importantly, both cyclin A and Chk2 are recruited to APAR bodies during MVM infection (2, 31), opening the possibility that viral sequestration of cyclin A or Chk2 might prohibit activation of FoxM1. In addition, CDK1 has been shown to phosphorylate FoxM1, which is critical for the interaction of PLK1 and FoxM1 (27). This interaction allows for direct phosphorylation and activation of FoxM1 by PLK1. Potentially, the lack of CDK1 kinase activity during MVM infection may prevent the activation of the known positive-feedback loop required for strong FoxM1 activation and execution of the mitotic program. It will be interesting to determine if MVM prevents the interaction of PLK1 and FoxM1. Although reconstitution of FoxM1 at the cyclin B1 gene promoter via dCas9 targeting elevated cyclin B1 RNA and protein levels during infection, it is not yet clear how constitutively active FoxM1 or the FoxM1 transactivation domain fused to dCas9 escapes MVM-induced inhibition. It is possible

that MVM prevents alleviation of FoxM1 autorepression, which may be circumvented by introduction of a constitutively active FoxM1 construct in which the inhibitory N-terminal domain has been deleted or of the transactivation domain without the presence of endogenous regulatory domains. Targeting the cyclin B1 gene promoter with either of the FoxM1-CRISPR/dCas9 constructs had no detectable effect on MVM expression or replication (data not shown), perhaps because of the modest increases in cyclin B1 levels which were achieved. Attempts to push MVM-infected cells into mitosis by supplying activated cyclin B1 have not yet been successful, which suggests that elevating cyclin B1 levels alone may not be sufficient to execute the mitotic program.

Numerous other viruses have been shown to affect the cell cycle during infection. HIV VPR inhibits the activation of the cyclin B1/CDK1 complex, possibly by binding to and altering the activity of the CDC25C phosphatase, which is required to remove the inhibitory phosphorylation of CDK1 (32–34). Human papillomavirus type 1 (HPV1), reoviruses, and SV40 also prevent activation of CDK1 by maintaining the inhibitory phosphorylation of CDK1 (35–37). HIV VPR alternatively upregulates the CDK inhibitor p21, which helps to maintain the inactive state of the cyclin B1/CDK1 complex (38). The agnoprotein of JC polyomavirus also induces p21 to inhibit the activation of the cyclin B1/CDK1 complex (39). HIV can also utilize its Tat protein to stimulate the expression of cyclin B1, which is thought to promote apoptosis, and then to target cyclin B1 for proteasomal degradation by binding to the N terminus of cyclin B1 (40). The E4 protein of HPV16 is able to sequester the cyclin B1/CDK1 complex in the cytoplasm, thus preventing its nuclear localization and activity (41). In the studies presented here, we demonstrated that MVM uses a different approach to prevent the activation of the cyclin B1/CDK1 complex, by depleting both the cyclin B1 protein and, preceding that loss, the cyclin B1 RNA.

MATERIALS AND METHODS

Cell lines, viruses, and virus infections. Murine A9 and human NB324K cells were propagated and wild-type MVMp produced as previously described (1). MVM1989 was propagated in 324K cells, and genome copies were quantified by Southern blotting (42). Infections were carried out at a multiplicity of infection (MOI) of 10 unless otherwise indicated. Lentivirus constructs designed to express cyclin B1 were generated by cotransfecting equal concentrations of HIV Gag/Pol, vesicular stomatitis virus glycoprotein G (VSV-G), and pINDUCER20 plasmids containing versions of the cyclin B1 gene, as described previously, into HEK293T cells (24, 43). Stable doxycycline-inducible A9 cell lines were generated by infection of A9 cells with a pseudotyped pINDUCER20 virus. Cell lines were selected with 800 μ g/ml of Geneticin (Gibco). pINDUCER20 lentivirus-transformed cell lines were induced with 500 ng/ml doxycycline hydrochloride (MP Biomedicals).

Cell synchronization and drug treatments. A9 cells were parasynchronized in G_0 by isoleucine deprivation, as previously described (44), and then infected with MVMp at the time of release into complete medium. Entry into S phase occurs approximately 12 h after release into complete medium (24). Virally infected cells harvested at 24 h thus represent an approximately 12-h transit into S phase. Doxorubicin (Sigma) was added at a concentration of 200 nM 15 h after release from isoleucine deprivation.

Antibodies. Commercially available antibodies against the indicated proteins were obtained from Abcam: H3K4me2 (ab32356), H3K9me3 (ab8898), RNA polymerase II S2 (ab5095), and RNA polymerase II S5 (ab5408). Rabbit IgG (2729S) and mouse IgG (5415S) were obtained from Cell Signaling. Antibody against cyclin B1 (05-373) was obtained from Millipore. Beta-actin antibody (MA515739) was obtained from Pierce. Antibodies to the following proteins were obtained from Proteintech: beta-actin (60008-1-Ig), B-Myb (18896-1-AP), FoxM1 (19147-1-AP), and NF-YA (12981-1-AP). FLAG antibody (F1804) was obtained from Sigma. NS1/2 (M55) antibody was described previously (1).

Plasmids. Murine wild-type cyclin B1 cDNA (Origene) was cloned into p3XFLAG-CMV 7.1 (Sigma). The FLAG-tagged wild-type cyclin B1 gene was cloned into pDONR221 (Invitrogen) and pINDUCER20 by use of BP and LR Clonase kits (Invitrogen), respectively. pINDUCER reagents were a gift from Guang Hu (NIH/NIEHS) (24, 43). pcDNA-dCas9-HA was produced by Charles Gersbach (Addgene plasmid 61355) (45). pgRNA-Humanized was produced by Stanley Qi (Addgene plasmid 44248) (46). pCMV-CD4 was a gift from Marc Johnson (University of Missouri).

Immunoblot analysis. Immunoblotting was performed as previously described (1). Protein concentrations were quantified by Bradford assay, and equal amounts of lysates were loaded for Western blot analysis, with actin levels serving as loading controls.

Cell cycle analysis. A9 cells were fixed in 70% ethanol overnight at 4°C. Cells were then pelleted, washed in phosphate-buffered saline (PBS), and resuspended in PBS containing 0.2 mg/ml RNase A (Roche) for 1 h at 37°C, after which propidium iodide (Sigma) was added to 40 μ g/ml. Flow cytometry was performed using an Accuri C6 flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (FlowJo, LLC).

TABLE 1 Primers used in this study

Primer set	Direction	Sequence (5'–3') ^a
Stability-cyclin B1 RNA-exon 1-exon 2	Forward	ATGGCGCTCAGGGTCACTAG
	Reverse	GGCACTCTTGCTGTAGCTC
Stability-cyclin B1 RNA-exon 6-exon 7	Forward	GCCTCTGCACTTCCTCCGTAGAGC
	Reverse	CCATTACCGTTGTCAAGAATTTTC
Nascent-cyclin B1 RNA-intron 1-exon 2	Forward	CTTTTCTTAGTTAGGTCCCGC
	Reverse	GTCTCAGCCCGGGCTTGGAAG
18S rRNA	Forward	TTGACGGAAGGGCACCACCAG
	Reverse	GCACCACCACCCACGGAATCG
GAPDH RNA	Forward	TTGTACAGGGAGGTGTGGA
	Reverse	GAATTTGCCGTGAGTGGAGT
ChIP- <i>Ccnb1</i> -promoter	Forward	GAACCTGGGATCGCGGGATCG
	Reverse	CTCCGCAGCACGCCGGGAGGA
ChIP- <i>Ccnb1</i> -promoter-S5	Forward	CGCGGGATCGCCAGGAAACG
	Reverse	CCGATTCGAGAAGACACCCTA
ChIP- <i>Ccnb1</i> -exon 2-S2	Forward	AACACGAAAATTAACGCAGAA
	Reverse	CCTTTTCAGAGGCACTCTTGC
Histone ChIP- <i>Ccnb1</i> -nonactive region-H3K4me2	Forward	CCTTCTAAGTTATGACACAGG
	Reverse	CAAGTTGACACACAAAACAG
Histone ChIP- <i>Ccnb1</i> -nonactive region-H3K9me3	Forward	CGTCATATGGCTGGTACCTGG
	Reverse	GCAGGGGAGGGAGAGCAGGAG
Histone ChIP- <i>Ccnb1</i> -active region	Forward	CTCGCGAGGTCAGGCTCTATG
	Reverse	CTGATATGCGTACTCCCCACAG

^aPrimers were obtained from Integrated DNA Technologies.

ChIP. A9 cells were cross-linked with 1% formaldehyde for 10 min at room temperature and then quenched with glycine (0.125 M). Cells were collected and lysed using ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, protease inhibitors). The lysates were sonicated using a Diagenode Bioruptor for 60 cycles (30 s on and 30 s off) and then immunoprecipitated with the indicated antibodies bound to protein A Dynabeads (Invitrogen). Samples were washed with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl, pH 8), and Tris-EDTA (TE) buffer (pH 7.5) and then eluted in SDS elution buffer (1% SDS, 0.1 M NaHCO₃). Following elution, chromatin-antibody complexes and input DNA were reverse cross-linked by heating and proteinase K digestion at 65°C overnight. The DNA was purified using a Qiagen PCR purification kit. ChIP assays were analyzed by quantitative PCR (qPCR) with iTAQ universal SYBR green master mix (Bio-Rad), using the primer sets shown in Table 1. Data are presented as the fold enrichment over input. Relative occupancy was calculated as previously described (17).

RPA. Total RNA was prepared using TRIzol reagent (Invitrogen), and an RNase protection assay (RPA) was performed as previously described (47). Murine cyclin B1 cDNA was obtained from Origene. Nucleotides 1 to 180 of the murine cyclin B1 gene were cloned into pGEM3Z to make an antisense probe (2). 3XF-Cyclin B1, murine beta-actin, and murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences were also cloned into pGEM3Z to make antisense probes.

Nascent RNA and RNA stability assays. For nascent RNA assays, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) was added at 40 μg/ml, as previously described (44), at 24 h postinfection and then incubated for 3 h. Following DRB treatment, cells were washed twice with PBS and incubated in complete medium for the indicated time points (48) prior to extraction.

For RNA stability assays, DRB was added at 40 μg/ml at 20 h postinfection. Cells were harvested every 2 h, as indicated.

At the indicated time points, total RNA was prepared using TRIzol reagent (Invitrogen), and cDNA was generated from 1 μg of RNA by using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). Quantitative PCR was performed with iTAQ universal SYBR green master mix (Bio-Rad). Nascent RNA generation data are presented as expression changes relative to expression at T0. Stability data were plotted on a semilogarithmic scale and are presented as percentages of mRNA relative to the level at T0, as previously reported (22). The 18S rRNA gene was used to normalize qPCR data.

dCas9-FoxM1 fusion assay. Guide RNAs for the cyclin B1 gene promoter were designed by use of the CRISPR Design tool (crispr.mit.edu) and initially cloned into pgrRNA-Humanized (Addgene). A con-

stitutively active FoxM1 construct with the N terminus deleted (Δ N-FoxM1) (19) was expressed directly from pcDNA, or it and a phosphomimetic FoxM1 transactivation domain (18) were fused to the C terminus of dCas9 in pcDNA-dCas9 for targeting prior to expression. Cells were cotransfected with cyclin B1 guide RNAs (to target the dCas9 vectors [or empty vector] to the cyclin B1 gene promoter), dCas9- Δ N-FoxM1 or dCas9-FoxM1-phospho-TAD, and pCMV-CD4 during parasynchronization. Following parasynchronization, cells were released into complete medium and infected at an MOI of 10. Cells were harvested at 30 h postinfection and positively selected by CD4 expression by use of a human CD4⁺ T cell enrichment kit (EasySep).

Statistical analysis. Real-time qPCR data were analyzed using Microsoft Excel, and statistical analysis was performed in GraphPad Prism (GraphPad Software).

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