Differential Mechanisms for the Involvement of Polyamines and Hypusinated eIF5A in Ebola Virus Gene Expression

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ABSTRACT Polyamines and hypusinated eIF5A have been implicated in the replication of diverse viruses; however, defining their roles in supporting virus replication is still under investigation. We have previously reported that Ebola virus (EBOV) requires polyamines and hypusinated eIF5A for replication. Using a replication-deficient minigenome construct, we show that gene expression, in the absence of genome replication, requires hypusinated eIF5A. Additional experiments demonstrated that the block in gene expression upon hypusine depletion was posttranscriptional, as minigenome reporter mRNA transcribed by the EBOV polymerase accumulated normally in the presence of drug treatment where protein did not. When this mRNA was isolated from cells with low levels of hypusinated eIF5A and transfected into cells with normal eIF5A function, minigenome reporter protein accumulation was normal, demonstrating that the mRNA produced was functional but required hypusinated eIF5A function for translation. Our results support a mechanism in which hypusinated eIF5A is required for the translation, but not synthesis, of EBOV transcripts. In contrast, depletion of polyamines with difluoromethylornithine (DFMO) resulted in a strong block in the accumulation of EBOV polymerase-produced mRNA, indicating a different mechanism of polyamine suppression of EBOV gene expression. Supplementing with exogenous polyamines after DFMO treatment restored mRNA accumulation and luciferase activity. These data indicate that cellular polyamines are required for two distinct aspects of the EBOV life cycle. The bifunctional requirement for polyamines underscores the importance of these cellular metabolites in EBOV replication and suggests that repurposing existing inhibitors of this pathway could be an effective approach for EBOV therapeutics.

IMPORTANCE Ebola virus is a genetically simple virus that has a small number of proteins. Because of this, it requires host molecules and proteins to produce new infectious virus particles. Though attention is often focused on cellular proteins required for this process, it has recently been shown that cellular metabolites such as polyamines are also necessary for EBOV replication. Here we show that polyamines such as spermine and spermidine are required for the accumulation of EBOV mRNA and that eIF5A, a molecule modified by spermidine, is required for the translation, but not the production, of EBOV mRNAs. These findings suggest that effectively targeting this pathway could provide a biphasic block of EBOV replication.

KEYWORDS eIF5A, Ebola virus, hypusination, polyamines, spermidine

Ebola virus (EBOV) is a nonsegmented negative-sense RNA virus in the *Filoviridae* family and is one of the most deadly pathogens known, with fatality rates ranging from 40 to 90%. The EBOV genome is limited in size, carrying only seven genes (encoding NP, VP35, VP40, GP, VP30, VP24, and L), and it is widely understood that EBOV relies on host proteins and molecules for its replication (1). Upon entering a cell during infection, the EBOV polymerase (L), viral proteins VP30 and VP35, and the

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nucleoprotein (NP)-encapsidated genome are released into the cell. These viral components then transcribe and replicate the EBOV genome before assembly and egress of a new viral particle. Polyamines are host molecules that have been broadly implicated in the replication of many diverse viruses (2), including filoviruses (3); however, there is limited understanding of how they are important for viral infection. These cellular cofactors are potential targets for the development of antiviral therapeutics (1).

The polyamines putrescine, spermidine, and spermine are small, positively charged molecules found in mammalian cells and are involved in numerous cellular functions, including protein synthesis, DNA and RNA structure, protein-RNA interactions, and gene expression (reviewed in references 2, 4, 5, 6, and 7). The cellular concentrations of polyamines are tightly regulated by the enzymes in their biosynthetic pathway and can be pharmacologically blocked via the drug difluoromethylornithine (DFMO), which targets ornithine decarboxylase (ODC), a key regulatory enzyme in the pathway (8).

Downstream of polyamine synthesis, the polyamine spermidine is used in the activation of translation factor eIF5A, called hypusination. Hypusination is achieved through a two-enzyme cascade, where an aminobutyl moiety from spermidine is first covalently attached to lysine 50 of eIF5A through the action of deoxyhypusine synthase (DHS) and then hydroxylated by deoxyhypusine hydroxylase (DOHH) to form the fully hypusinated eIF5A (9). This pathway can be pharmacologically targeted by treating cells with the DHS inhibitor *N*1-guanyl-1,7-diamineheptane (GC7) or the DOHH inhibitors ciclopirox (CPX) or deferiprone (10). Hypusination is essential for the function of eIF5A, and eIF5A is the only hypusinated protein in the cell. In the cell, eIF5A has been implicated in translation elongation of "hard-to-translate" sequences, such as polyproline stretches and other tripeptide motifs, as well as more broadly in translation termination (9, 11).

In addition to their roles in cellular processes, polyamines and hypusinated eIF5A have been broadly implicated in the replication of diverse viruses, including RNA viruses such as EBOV and Zika virus, DNA viruses such as herpes simplex virus 1 and vaccinia virus, and retroviruses such as HIV, among others (2). There is limited understanding of the mechanisms in which viruses require polyamines and/or hypusinated eIF5A. For example, polyamines have been shown to be required for chikungunya virus (CHIKV) replication at the level of translation and RNA-dependent RNA polymerase activity in infected cells (12, 13). Previous work from our lab has shown that polyamines and hypusinated eIF5A are required for Ebola virus (EBOV) gene expression (3). Here we investigated whether there is a single mechanism in which polyamines are required through the hypusination of eIF5A or whether there are distinct requirements of polyamines and hypusinated eIF5A. We hypothesized that hypusinated eIF5A is required at the level of translation, due to its function as a translation factor, and that polyamines may be required at multiple stages of gene expression due to the diversity in the cellular roles they play.

Toward understanding the mechanism in which hypusinated eIF5A is required for EBOV gene expression, we evaluated several aspects of gene expression, including reporter gene transcription, translation, and protein stability, as possible hypotheses. Our results support a mechanism in which hypusinated eIF5A is required for the translation of EBOV transcripts, including minigenome reporter genes, as well as authentic EBOV mRNAs expressed from a tetracistronic minigenome. We also investigated whether polyamines were similarly required for EBOV mRNA translation, presumably through the requirement of spermidine for hypusination, or whether there was a distinct requirement for polyamines leading to an additional mechanism of inhibition. The results indicate that polyamines are required for transcription of EBOV minigenome mRNAs, representing a requirement of polyamines that is different from hypusinated eIF5A. These data suggest that cellular polyamines support two distinct aspects of the EBOV life cycle, first, directly for EBOV gene transcription/mRNA accumulation, and second, indirectly through the incorporation of spermidine into the translation factor eIF5A to support translation of EBOV mRNAs, identifying a multifaceted approach at potential avenues for EBOV therapeutics.

FIG 1 Effect of hypusination inhibitors on EBOV minigenome reporter expression and stability. (A) Schematic of a replicating and transcribing minigenome. A plasmid carrying the minigenome construct is transcribed by T7 polymerase to provide template minigenomes which are transcribed and replicated by the EBOV polymerase complex (also provided by T7-driven expression plasmids). Minigenome reporter gene mRNA is transcribed by the EBOV polymerase complex (L, NP, VP30, and VP35) and subsequently translated by host machinery. The minigenome construct is also replicated by the EBOV polymerase replication complex (L, NP, and VP35), increasing the minigenome template available for transcription and further replication. (B) EBOV minigenome-driven luciferase expression (in relative luminescence units [RLU]) in the presence (gray bar) and absence (black bar) of GC7. Data are normalized relative to non-drug-treated cells. Error bars represent standard errors of the means (SEM) from three independent experiments. (C) Schematic of a replication-deficient, transcription-competent minigenome. This construct has a deletion in the trailer region removing the antigenome replication promoter and therefore cannot complete the genome replication cycle. The mRNA from this construct is a result of only minigenome reporter gene transcription with no amplification of minigenome template from genome replication. (D) EBOV replication-deficient minigenome-driven luciferase expression (nonreplicating [NR]) (in relative luminescence units) in the presence (gray bar) and absence (black bar) of GC7. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (E) Decay of EBOV minigenome-driven luciferase expression (in relative luminescence units), comparing GC7-treated cells (blue) and non-drug-treated cells (yellow) in the presence of CHX and the absence of CHX (GC7-treated cells, red; non-drug-treated cells, green). Data are normalized relative to 0 h after CHX treatment. Error bars represent ranges from two independent experiments. *, $P < 0.05$; **, $P < 0.01$ (ratio paired *t* test).

RESULTS

EBOV gene expression requires hypusinated eIF5A. We previously reported that EBOV minigenome luciferase reporter gene expression requires hypusinated eIF5A (3). The data suggested that eIF5A dependence was due to the requirement of hypusinated eIF5A for the accumulation of VP30 protein, which is a viral polymerase cofactor essential for EBOV gene transcription. In the course of studying the sensitivity of plasmid-expressed VP30, we noted that VP30 protein expressed from a pTM1 plasmid was not sensitive to a depletion of hypusinated eIF5A, and VP30 protein accumulation was no longer reduced (see the correction of reference 3). This finding allowed us to ask whether EBOV gene expression was altered under conditions where VP30 levels were not repressed.

We assessed the requirement of hypusinated eIF5A for EBOV gene expression when VP30 protein accumulates (via expression from a pTM1 expression plasmid) using the EBOV minigenome system as previously described (3, 14) (Fig. 1A). GC7 was chosen as a representative inhibitor for hypusination due to its robust and specific reduction of EBOV minigenome luciferase activity with no effect on the green fluorescent protein (GFP) control, as shown previously (3). Cells were treated with GC7 for 24 h and transfected with minigenome system components, and luciferase expression was measured at 24 h posttransfection. We found that gene expression from the EBOV minigenome was still blocked even when VP30 accumulated, with an 85% reduction in luciferase activity, following GC7 treatment, confirming our initial observation that EBOV minigenome expression is dependent on hypusinated eIF5A (Fig. 1B). This led us to investigate the additional mechanisms in which EBOV gene expression requires hypusinated eIF5A.

The reduction of reporter gene expression by inhibition of eIF5A hypusination is independent of genome replication. We then sought to define whether the defect in EBOV minigenome activity when hypusination is blocked was due to deficiencies in gene expression (transcription/translation of a reporter gene) when genome replication is disrupted. We investigated whether hypusinated eIF5A was required for reporter gene expression of a nonreplicating minigenome construct where the last 25 nucleotides of the trailer were deleted to remove the antigenomic replication promoter. This minigenome construct allows us to interrogate transcription/translation independently of replication. "Replication" (i.e., antigenome synthesis) will still occur, as the leader promoter is intact, but multiround replication will not occur and thus will not influence transcription/translation. Therefore, the luciferase expression from this construct is a result of only viral gene transcription by the EBOV RNA-dependent RNA polymerase (L) (Fig. 1C). As shown in Fig. 1D, expression of the minigenome luciferase reporter from the nonreplicating, transcription-competent minigenome was significantly blocked by inhibition of hypusination. Although we cannot rule out an additional effect on viral replication, these results indicate that there is a defect in viral gene expression at the level of transcription, translation, or protein stability.

Reporter protein stability is not affected by GC7 treatment. To determine whether there is a defect in reporter protein stability in the presence of hypusination inhibitors, we conducted a pulse-chase assay in the presence and absence of cycloheximide (CHX). Cells were treated with GC7 for 24 h, transfected with minigenome components, and then pulsed with either CHX or dimethyl sulfoxide (DMSO). Luciferase assays were conducted at 0, 6, and 12 h after addition of CHX. There was no difference in decay of the luciferase signal in CHX-treated samples with and without GC7 (Fig. 1E). These results indicate that GC7 does not alter the stability or activity of the luciferase reporter protein, suggesting that the lack of reporter activity was due to changes at the level of mRNA accumulation or translation.

The quality and quantity of luciferase reporter mRNA are unaffected by GC7 inhibition of hypusination. To understand whether GC7 altered viral mRNA accumulation, we next quantified luciferase mRNA levels via Northern blotting. To measure luciferase mRNA levels, RNA was extracted at 24 h posttransfection and analyzed via Northern blotting (Fig. 2A). The luciferase mRNAs in GC7-treated and nontreated samples appeared to be highly similar, running at approximately the same molecular weight and accumulating to similar levels (Fig. 2B). When mRNA levels were quantified over three independent experiments, the levels in GC7-treated and untreated samples were not significantly different (Fig. 2C).

To corroborate our findings, we tested another small-molecule inhibitor of hypusination, ciclopirox (CPX). CPX is an iron chelator with multiple molecular targets (15), one of which is deoxyhypusine hydroxylase, which requires an iron cofactor. We found that inhibition of hypusination with CPX also results in a decrease of EBOV gene expression (Fig. 2D) (3) but not of control firefly luciferase (ffLuc) gene expression (Fig. 2E). We next measured EBOV minigenome-derived mRNA levels with and without CPX treatment. Northern blot analysis indicated that there was a slight but variable reduction in luciferase mRNA (Fig. 2F and G). We further compared the relative mRNA decrease with the decrease in luciferase activity to show that the decrease seen in mRNA was not commensurate with the decrease in activity (Fig. 2H), suggesting that the decrease in luciferase activity was mostly due to a reduction in translation, as was found with GC7 treatment.

To understand the diminished translation of the mRNA produced by the EBOV polymerase in the presence of GC7, we examined whether the mRNA could be properly translated if introduced into cells with functional hypusinated eIF5A. To test this, total RNA was extracted from cells transfected with minigenome components in the presence or absence of GC7 in the same manner as described above. This RNA was then transfected into fresh cells that were not treated with any compounds. At 24 h posttransfection, luciferase activity from cells transfected with the different sources of

FIG 2 Effect of hypusination inhibitor on minigenome reporter mRNA accumulation. (A) Representation of experimental setup. Cells were seeded on day 0, treated (or non-drug treated) on day 1, and transfected with the minigenome components on day 2, and on day 3 RNA was extracted from cells for Northern blot analysis or for transfection into fresh cells, followed by a luciferase assay on day 4. (B) Representative Northern blot of fLuc mRNA accumulation from a minigenome transfection in the presence or absence of GC7. ND, no drug. (B') Methylene blue staining for a representative Northern blot to show the amount of RNA loaded for each sample. Northern blot signals were normalized to the density of RNA bands from methylene blue staining. (C) Quantification of Northern blot results of three independent experiments measuring firefly luciferase mRNA levels with or without GC7. (D) EBOV minigenome-driven luciferase expression (in relative luminescence units) in the presence (gray bar) or absence (black bar) of cyclopirox (CPX). Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (E) pTM1-driven firefly luciferase activity measured with or without CPX as a control. (F) Representative Northern blot of fLuc mRNA accumulation from a minigenome transfection in the presence or absence of CPX. (F') Methylene blue staining for a representative Northern blot to show the amount of RNA loaded for each sample. Northern blot signals were normalized to the density of RNA bands from methylene blue staining. (G) Quantification of Northern blot results of three independent experiments measuring firefly luciferase mRNA levels with or without CPX. (H) Summary comparison of relative mRNA transcript levels and relative luciferase signals for both GC7 and CPX treatment. Data are normalized relative to non-drug-treated cells and are representative of an average of three independent experiments. (I) EBOV minigenome-driven luciferase expression (in relative luminescence units) from RNA isolated from cells in the presence (gray bar) or absence (black bar) of GC7. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (J) [35 S]methionine incorporation measuring global translation in the presence (gray bar) or absence (black bar) of GC7 treatment. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; NS, not significant (ratio paired t test).

RNA was measured. Regardless of the source of the mRNA (from cells treated with GC7 or nontreated), there was no difference in luciferase activity (Fig. 2I). Taken together, these data indicate that mRNA produced by the EBOV polymerase in GC7-treated cells is not inherently defective and can be translated when it is introduced into a cell with normalized levels of hypusinated eIF5A, suggesting that the defect is at the level of mRNA translation when hypusinated eIF5A is depleted.

We also wanted to verify that the defect in translation of minigenome-derived mRNA was not due to an overall defect in global translation. To test this, we conducted [35 S]methionine labeling of cells with and without GC7 treatment. Surprisingly, we found a slight increase in global translation of cells treated with 10 μ M GC7 for up to 48 h (Fig. 2J). These results support the notion that there is not an overall reduction in global translation, and the effect of inhibiting hypusination on the translation of minigenome-derived mRNA is distinct.

Hypusination is required for translation of EBOV minigenome-derived reporter mRNAs. The data presented thus far support a hypothesis in which the defect in EBOV minigenome reporter gene expression is a defect in the translation of the EBOV

FIG 3 Effect of hypusination inhibitor on expression of firefly luciferase mRNA from alternative sources. (A) Luciferase expression from several expression vectors (in relative luminescence units) in the presence (gray bars) and absence (black bars) of GC7. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (B) Schematic of ffLuc mRNA synthesis from 3E5E construct. (C) Schematic of ffLuc mRNA synthesis expressed directly from vector via *in vitro* transcription by T7 RNA polymerase and poly(A) tailing. (D) Luciferase expression from *in vitro*-transcribed firefly luciferase mRNA transfected into cells from a 3E5E or control ffLuc direct expression vector (in relative luminescence units) in the presence (gray bars) and absence (black bars) of GC7. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. NS, not significant (ratio paired *t* test).

minigenome firefly luciferase (ffLuc) mRNA. The ffLuc mRNA produced from the EBOV minigenome system contains the ffLuc open reading frame (ORF) as well as upstream and downstream EBOV untranslated regions (UTRs) flanking the reporter gene. Upstream of the ORF is the EBOV leader and NP 5' UTR. Downstream of the ORF is a partial L 3' UTR and EBOV trailer. We sought to understand whether the hypusination requirement for translation was specific to the ffLuc gene sequence (ORF), whether it was due to the flanking EBOV UTRs, or whether it was due to the viral polymerase. Previous data indicate that the defect in minigenome reporter mRNA translation is not due to the reporter gene, as we have shown that a minigenome encoding *Renilla* luciferase (rLuc) was similarly affected by a block in hypusination with GC7 treatment (3). To address whether the EBOV UTRs were conferring sensitivity, we measured luciferase activity generated from several different expression vectors expressing firefly luciferase (in the absence of any EBOV sequence) (for complete vector sequences, see Fig. S2 to S4 in the supplemental material) in the presence or absence of GC7 treatment. Each of these expression vectors will yield mRNAs with 5' and 3' UTRs unique to each expression vector. Of the expression vectors tested (pTM1, pMIR, and pCDNA3), none showed a significant change in ffLuc activity with GC7 treatment (Fig. 3A), indicating that general translation of ffLuc mRNA does not require hypusinated eIF5A. Together, these data suggest that the defect in gene expression from the minigenome construct is EBOV specific and independent of the reporter gene.

FIG 4 Effect of hypusination inhibitor on EBOV genes using a tetracistronic minigenome. (A) Cartoon representation of the tetracistronic minigenome construct (p4cis). (B) EBOV minigenome-driven *Renilla* luciferase (rLuc) expression (in relative luminescence units) in the presence (gray bar) and absence (black bar) of GC7. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from four independent experiments. (C) Representative Northern blot of rLuc mRNA with or without GC7 treatment. (D) Quantification of three independent Northern blots analyzing rLuc mRNA accumulation. Data are normalized relative to non-drug-treated cells. (E) Representative Western blot analysis of EBOV proteins and hypusine with or without GC7 treatment. (F) Quantification of four independent experiments analyzing EBOV protein accumulation. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from four independent experiments. *, $P < 0.05$; NS, not significant ratio paired t test.

To further understand the context in which mRNA transcribed from the EBOV minigenome requires hypusine for translation, we next investigated whether the mRNA translation defect was due to EBOV polymerase-based transcription (as is depicted in Fig. 3B) or whether the defect was still present if the same mRNA was transcribed from a T7 polymerase (as depicted in Fig. 3C). To test this, the EBOV minigenome fLuc construct with EBOV UTRs was cloned into a pCDNA3 vector in positive-sense orientation for *in vitro* transcription (Fig. 3C; for full plasmid sequence and map, see Fig. S1). The construct was *in vitro* transcribed, capped, and polyadenylated, and the resulting mRNA was transfected into cells. As a control, we also cloned and expressed only the ORF of fLuc from the same vector. Cells were treated with GC7 for 24 h, transfected with either the 3E5E-fLuc mRNA or the control fLuc mRNA, and assayed for luciferase activity 24 h later. We found no specific reduction in 3E5E fLuc activity compared to the control in GC7-treated cells (Fig. 3D). These data indicate that these T7 polymerase-derived mRNAs do not require hypusinated eIF5A for translation and that the eIF5A translation requirement is conferred by EBOV polymerase transcription.

Other EBOV genes also require hypusinated eIF5A for expression. The data thus far suggest that all mRNAs transcribed by the EBOV polymerase might be sensitive to levels of hypusinated eIF5A. To investigate this possibility, we assessed the accumulation of additional EBOV proteins using a tetracistronic minigenome (p4cis) which encodes 3'-leader-*Renilla* luciferase-VP40-GP-VP24-trailer-5' (Fig. 4A) (16). We reasoned that if transcription from the EBOV polymerase conferred eIF5A dependence, we would observe multiple EBOV-transcribed genes that had lower protein accumulation. Cells were treated with GC7 for 24 h, transfected with the p4cis minigenome construct along with the other system components, and assayed for luciferase activity and protein

accumulation (by Western blotting) at 24 h posttransfection. Consistent with our ffluc minigenome reporter, rLuc activity was still significantly reduced in the presence of hypusination inhibitors (Fig. 4B), even though the mRNA was present at levels similar to those in the nontreated control (Fig. 4C and D, Northern blotting). We then assessed the accumulation of the three EBOV proteins, VP40, GP, and VP24, that were derived from EBOV polymerase-based transcription. VP40 protein accumulation was significantly reduced when hypusination was blocked and VP24 was also slightly affected (Fig. 4E and F), and we were unable to reliably detect GP protein from the first round of transfections. These data suggest that some, if not all, EBOV-transcribed mRNAs require hypusinated eIF5A for translation.

Polyamines are required at a stage of gene expression distinct from hypusinated eIF5A. We next determined whether blocking polyamine synthesis (upstream of hypusination) provided the same block in EBOV mRNA translation as was seen when hypusination was interrupted. We have previously shown that blocking polyamine synthesis with DFMO results in a strong and significant defect in EBOV minigenome expression (Fig. 5A), without having any effect on a GFP control (3). We reasoned that EBOV may also require polyamines for translation of its transcripts, presumably through the requirement of spermidine for hypusination. First, we assessed whether mRNA from cells treated with the ornithine decarboxylase inhibitor DFMO would be translated if transfected into fresh cells containing normal levels of polyamines, similar to RNA transfection experiments conducted with the hypusination inhibitor GC7. Cells were treated with DFMO for 24 h and transfected with the EBOV minigenome components, and then either the cells were subjected to a luciferase assay to verify reduced expression or total RNA was collected and purified to transfect fresh cells (Fig. 2A). As previously shown, DFMO significantly reduces luciferase activity from the minigenome reporter by about 90% (Fig. 5A) but does not affect that from a control ffluc (Fig. 5B) (3). Unlike what we found with the hypusination inhibitor GC7, we found that RNA samples from lysates with reduced luciferase activity following DFMO treatment still had reduced luciferase activity (reduced by 80%) when transfected into fresh cells (Fig. 5C). This suggests that unlike in the GC7 experiments, where the RNA was present and intact but not translated, in DFMO-treated cells either the EBOV polymerase did not synthesize mRNA or the synthesized mRNA was not translatable. To differentiate between these two possibilities, we conducted Northern blot analysis to measure EBOV-transcribed mRNA levels. We found that the level of EBOV minigenome-derived ffluc mRNA from DFMO-treated cells was significantly reduced compared to that from nontreated cells (Fig. 5D and E), indicating that the block in gene expression is, at least in part, at the transcriptional level.

We then sought to analyze whether the defect in mRNA accumulation was still present in the absence of genome replication, which would indicate a defect in gene transcription. To investigate this, we assessed whether minigenome-derived luciferase activity was still reduced in DFMO-treated cells when a replication-deficient, transcription-competent minigenome was used (Fig. 1C). As shown in Fig. 5F, luciferase expression from the replication-deficient minigenome was still significantly blocked by inhibition of polyamine synthesis. Although we do not rule out the possibility of an additional defect in genome replication, these results indicate that the reduction of viral gene expression occurs at the level of transcription when polyamine synthesis is blocked by DFMO.

Although there is a strong defect in the accumulation of minigenome mRNA, we also wanted to investigate whether there could be an additional role for polyamines in the translation of minigenome mRNA. To test this, we transfected ffluc mRNA *in vitro* transcribed by a T7 RNA polymerase using a plasmid carrying the EBOV minigenome luciferase construct (containing EBOV UTRs). The ffluc mRNA was also capped and polyadenylated during *in vitro* synthesis. The mRNA was then transfected into cells that were treated with DFMO (or not drug treated) for 24 h. For comparison, we also used *in vitro*-transcribed ffluc mRNA that was not flanked by EBOV UTRs. Interestingly, there seems to be an overall effect of DFMO on translation of both the 3E5E-derived mRNA

FIG 5 Effect of polyamine synthesis inhibitor DFMO on minigenome reporter expression. (A) EBOV minigenome-driven luciferase expression (in relative luminescence units) in the presence (gray bar) and absence (black bar) of DFMO. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from four independent experiments. (B) Control ffLuc activity expressed from a pTM1 plasmid with or without DFMO. (C) RNA was isolated from cells with (gray bar) or without (black bar) DFMO treatment and then transfected into fresh cells. EBOV minigenome-driven luciferase expression (in relative luminescence units) was measured at 24 h posttransfection of RNA. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from four independent experiments. (D) Representative Northern blot showing ffLuc mRNA accumulation with or without DFMO. ND, no drug. (D') Methylene blue staining for representative Northern blot to show amount of RNA loaded for each sample. Northern blot signals were normalized to the density of RNA bands from methylene blue staining. (E) Quantification of Northern blot data from five independent experiments. (F) Nonreplicating (NR) EBOV 3E5E minigenome-driven luciferase expression (in relative luminescence units) in the presence (gray bar) and absence (black bar) of DFMO. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (G) Luciferase expression from T7-driven *in vitro*-transcribed firefly luciferase mRNA transfected into cells from a 3E5E or control ffLuc direct expression vector (in relative luminescence units) in the presence (gray bars) and absence (black bars) of DFMO. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (H) [35 S]methionine incorporation assay measuring global translation in cells treated with DFMO for 48 h or not drug treated. *, $P < 0.05$; **, $P < 0.01$; NS, not significant (ratio paired t test).

and the control ffLuc mRNA, with a reduction in expression of about 50% in the presence of DFMO (Fig. 5G). This effect was mimicked in a [35 S]methionine incorporation assay for total cellular translation, where DFMO reduced translation by about 10 to 20% (Fig. 5H). These data indicate that blocking polyamine synthesis with DFMO may also affect overall translation, not specific to EBOV mRNAs, whereas a block in hypusination with GC7 was specific to EBOV-derived mRNA translation.

We further wanted to demonstrate that the defects in EBOV mRNA accumulation were specific to a depletion in polyamines and not an off-target effect of DFMO treatment. To address this, we rescued DFMO treatment by supplementing with exogenous polyamines under conditions where the DFMO phenotype was exacerbated (5 mM DFMO). Cells were treated with DFMO for 24 h and transfected with minigenome components for 1 h, followed by supplementation of the medium with exogenous polyamines. At 24 h posttransfection, cells were either subjected to a luciferase assay

FIG 6 Rescue of DFMO treatment with exogenous polyamines. (A) EBOV minigenome-driven luciferase expression (in relative luminescence units) in the presence and absence of DFMO with and without exogenous polyamine supplementation. Data are normalized relative to non-drug-treated cells. Error bars represent SEM from three independent experiments. (B) Left panel, representative Northern blot showing ffLuc mRNA accumulation with or without DFMO and exogenous polyamines. Right panel, methylene blue staining for a representative Northern blot to show amount of RNA loaded for each sample. Northern blot signals were normalized to the density of RNA bands from methylene blue staining. (C) Quantification of Northern blot data from three independent experiments. *, $P < 0.05$; ***, $P < 0.001$; NS, not significant (ratio paired t test).

or RNA was harvested for Northern blotting. Cells which were treated with DFMO and then supplemented with exogenous polyamines showed almost complete rescue of both luciferase activity (Fig. 6A) and luciferase mRNA accumulation (Fig. 6B and C). These results indicate that the DFMO phenotype was due to a depletion of polyamines and support the hypothesis that polyamines are required for EBOV mRNA accumulation.

DISCUSSION

Previous work from our lab has shown that both polyamines and hypusinated eIF5A are required for EBOV gene expression and that this requirement is due to a defect in VP30 protein accumulation (3). The data presented here demonstrate that upon VP30 rescue via expression from an alternative expression vector, EBOV gene expression is still significantly reduced when hypusination is blocked with GC7 treatment. These data led us to investigate the additional mechanisms in which hypusination is required for EBOV gene expression.

Here we show that blocking polyamine synthesis at the level of ODC with DFMO and blocking hypusination at the level of DHS with GC7 have different effects on EBOV mRNA accumulation and function, with each approach leading to an inhibition of EBOV gene expression. The different steps in the EBOV life cycle targeted by these two

FIG 7 Model depicting mechanism of polyamine and hypusinated eIF5A requirements for EBOV gene expression. During a minigenome assay or EBOV infection, the polymerase complex assembles (L, purple; VP30, blue; VP35, yellow), and NP (green) encapsidates the genome. The polymerase complex then transcribes EBOV genes, in this case, the minigenome luciferase reporter gene. The mRNA is then translated by the host translation machinery. Upon treatment with small-molecule inhibitors of polyamines synthesis (DFMO) or hypusination (GC7), there is an overall reduction in luciferase protein amount, as measured by luciferase assay or Western blotting. The mechanisms by which this reduction occurs are distinct for the two inhibitors: DFMO inhibition of polyamine synthesis blocks minigenome expression at the level of transcription, as evidenced by a reduction in luciferase mRNA; GC7 inhibition of hypusination blocks at the level of mRNA translation, as evidenced by mRNA accumulation but a reduction in luciferase protein and activity.

approaches are depicted in our model in Fig. 7. In this model, the EBOV proteins involved in transcription (L, VP30, VP35, and NP) assemble, transcribe, and replicate the minigenome. The mRNA transcribed from the EBOV polymerase complex is routed for translation by the host protein synthesis machinery and results in the accumulation of luciferase protein. Blockade of ODC function to reduce intracellular polyamine levels results in a defect in mRNA accumulation. In contrast, blockade of hypusination does not alter mRNA accumulation but limits the effective translation of mRNA transcribed by the EBOV polymerase. Targeting either pathway pharmacologically results in the same apparent overall phenotype: reduced expression and activity of the minigenome reporter.

We carefully titrated and timed the use of the different small molecules so that different effects could be identified. We used short treatments with DFMO targeting the key regulatory enzyme in the polyamine synthesis pathway (ODC) to reduce intracellular polyamine levels without reducing cellular levels of hypusinated eIF5A. The loss of mRNA accumulation with DFMO shows that polyamines are important factors in EBOV polymerase activity. We hypothesize that this is due to a defect in the function of the proteins involved in replication and transcription, which could be a result of improper accumulation/assembly of viral components (VP30, VP35, NP, or L) or polymerase processivity. Polyamines have been implicated in the polymerase function for other viruses as well, for example, Semliki Forest virus and chikungunya virus (12, 17). Further work aims to elucidate the detailed mechanisms underlying this interaction.

The eIF5A dependency of EBOV tracks to mRNA translation and not mRNA accumulation. There are no previous examples of eIF5A being involved in promoting the translation of viral mRNAs, but the notion of virus mRNA translation being distinct from the cellular process is a well-established concept. A common way for viruses to gain access to the ribosome is through *cis*-acting sequences in the viral mRNAs which function to recruit viral and/or host factors to promote efficient translation. This is true for adenoviruses, where sequences in their 5' UTRs promote ribosomal shunting (18,

19), for rotavirus mRNAs, which recruit viral protein NSP3 to bind host translation initiation factor eIF4F (20), and for picornaviruses, which have an internal ribosome entry site sequence in their 5' UTRs to promote translation initiation when cap-dependent translation of cellular mRNAs is blocked (21).

Our results suggest that there is not a sequenced-based *cis*-acting element for EBOV mRNAs, as only virally derived luciferase mRNAs are sensitive to a block in hypusination, and when the same RNAs (by sequence) are transcribed by T7 RNA polymerase *in vitro*, they are not sensitive to a block in hypusination. These data are reminiscent of findings with vesicular stomatitis virus (VSV), where transcription from the viral genome enables efficient translation while host protein synthesis is decreased (22). That study demonstrated that the translation efficiency of the enhanced GFP (eGFP) mRNA was different when it was transcribed by the VSV RNA-dependent RNA polymerase than when it was transcribed by the host polymerase II (Pol II). Similarly, our work suggests that there is some element conferred by the EBOV polymerase that causes these mRNAs to require hypusinated eIF5A for translation. Understanding the precise details by which EBOV mRNAs require eIF5A for translation will be of great interest, as it may point to novel modifications to mRNAs made by the EBOV polymerase or novel mechanisms in which eIF5A aids in translation of mRNAs synthesized in the cytoplasm.

There is also relatively little known about how EBOV subverts the cellular translational machinery to promote translation of its mRNAs during infection. The EBOV polymerase makes mRNAs that are capped and polyadenylated, similar to host mRNAs and other negative-sense nonsegmented RNA virus mRNAs. What is unusual, however, is that EBOV produces mRNAs with long 5' and 3' UTRs, many of which contain upstream ORFs (uORFs). It has been shown, specifically for L polymerase mRNA, that a uORF suppresses translation of the primary ORF, and then, under conditions of cellular stress, phosphorylation of eIF2 α allows ribosomal scanning of the messenger to the primary ORF, promoting the translation of L mRNA (23). Interestingly, a separate study showed that in persistently infected cells, EBOV translation appeared to be inhibited in the presence of eIF2 α phosphorylation and to be stimulated following mitogen-activated protein kinase (MAPK) activation (24). Promoting translation through an eIF5A-dependent mechanism may be another strategy for EBOV to support its replication during infection.

Although we have not defined a specific mechanism of how EBOV mRNAs require eIF5A, our understanding of the myriad of roles for hypusinated eIF5A within the cell has expanded during recent years. Originally identified as a translation initiation factor stimulating dipeptide synthesis, the suggested role for eIF5A in mRNA translation has been modified to include it also being important for the translation of "hard-to-translate" regions such as polyproline stretches (9, 25, 26) and for translation termination (11). It was surprising that our global translation assays indicated an increase in cellular translation after GC7 treatment, in contrast to other published data (27). We attribute these discrepancies to a difference in cell lines (HEK293T versus BSR-T7), drug concentrations (100 μ M versus 10 μ M), and/or techniques used to measure [35 S]methionine incorporation (trichloroacetic acid [TCA] precipitation versus SDS-PAGE and autoradiography), thus not allowing for a direct comparison of results. Our data also demonstrate that protein synthesis inhibition through GC7 treatment appears to be selective to the virally derived luciferase mRNA, as T7- and RNA Pol II-derived luciferase accumulates efficiently (Fig. 3A and B) and global translation is not reduced. This indicates that there is not a requirement of eIF5A for a sequence-based elongation defect, as could be possible based on several "hard-to-translate" tripeptide motifs such as PPG and RDK. A role for eIF5A in stimulating translation via a structural element has yet to be described.

Our data presented here demonstrate that although polyamines and hypusinated eIF5A are both required for EBOV gene expression, they are required at distinct stages within this process. Probing these pathways pharmacologically suggests that EBOV replication can be subverted at the levels of both viral transcription, utilizing DFMO to block polyamine synthesis, and translation of viral mRNAs, using GC7 to block hypusi-

nation. Further work aims to identify the specific requirements of polyamines for EBOV transcription as well as the elements of EBOV mRNAs requiring hypusinated eIF5A for translation. More-detailed mechanistic insight into these processes will contribute to the development of therapeutic strategies to combat EBOV.

MATERIALS AND METHODS

Cells and reagents. All experiments were carried out in BSR T7/5 cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and L-glutamine (supplemented DMEM). The cells were grown in an incubator at 37°C under 5% CO₂.

N1-guanyl-1,7-diamineheptane (GC7) (10 μ M in H₂O) was purchased from LGC Biosearch Technologies. As recommended by the manufacturer, GC7 was used together in cell culture with 0.5 mM aminoguanidine to prevent destruction by monoamine oxidase (in H₂O). Ciclopirox olamine (3 μ M in water) was purchased from Sigma. 2-Difluoromethylornithine (DFMO) (500 μ M or 5 mM [for rescue experiments] in water) was a kind gift from Patrick Woster. Exogenous polyamines (10 $\times$ in medium from 1,000 $\times$ polyamine supplement) was purchased from Sigma.

Antibodies for immunoblots were used at the following dilutions: rabbit anti-VP30 N-terminal (prepared by GenScript), 1:5,000; rabbit antihypusine (EMD Millipore), 1:1,000; rabbit anti-VP40 (IBT), 1:1,000; rabbit anti-VP24 (IBT), 1:1,000; rabbit anti-*Renilla* luciferase (Abcam), 1:2,000; rabbit anti-hsp90 (Santa Cruz), 1:1,000; mouse anti- β -actin C4 (Santa Cruz), 1:1,000; and IRDye donkey anti-mouse 680 and donkey anti-rabbit 800 secondary antibodies (Li-Cor Biosciences), 1:10,000.

Minigenome assay. Cells seeded in a 24-well plate were treated with small-molecule inhibitors at the indicated concentrations (diluted in supplemented DMEM) for 24 h. Cells were then transfected with pTM1 plasmids containing the components of the EBOV polymerase complex under control of a T7-driven promoter (L, 115 ng; VP30, 145 ng; VP35, 115 ng; and NP, 235 ng), along with a reporter construct (p2,0-3E5E-luc [28], 1,400 ng) encoding firefly luciferase (ffLuc), using Lipofectamine 3000 (Invitrogen); these are collectively defined here as the minigenome system components. At 1 h post-transfection, drugs were added back into the transfection reaction at a 2 $\times$ concentration in supplemented DMEM to achieve the original dilution concentration. At 24 h posttransfection, cells were lysed with the Nanolight firefly luciferase assay reagent (Nanolight Technologies), and ffLuc activity was measured using a Tecan Spark multimode reader. Alternatively, either cells were lysed with NP-40 lysis buffer and the lysate was subjected to immunoblotting or RNA was isolated using TRIzol extraction. Luciferase will be expressed only if the components of the EBOV polymerase complex are expressed from the pTM1 support plasmids (VP30, VP35, NP, and L) through T7-driven transcription and translated by the host translational machinery. The polymerase complex is then able to transcribe ffLuc mRNA from the minigenome construct (which is flanked by the EBOV leader and trailer regions), and ffLuc is subsequently translated by host machinery. The minigenome RNA template is also replicated by the polymerase complex, which amplifies reporter gene expression. The resulting reporter gene expression represents both EBOV transcription and replication. For the tetracistronic minigenome (p4cis) or replication-deficient minigenome, the same protocols were used, replacing the 3E5E plasmid with the respective minigenome plasmid. The p4cis minigenome encodes *Renilla* luciferase, and therefore the Renilla-Glo luciferase assay system (Promega) was used to detect luciferase activity. The replication-deficient minigenome encodes ffLuc. For individual plasmid transfections, 500 ng of the indicated plasmid DNA was transfected per well (24-well plate).

RNA analyses. Total RNA was extracted and isolated from cells using TRIzol reagent (Thermo Fisher) according to manufacturer's specifications.

Northern blot analysis was conducted according to the manufacturer's recommendations using the Northern Max Gly kit (Ambion). RNA was glyoxal treated, and equal amounts (quantified with a NanoDrop instrument) were loaded onto a 1% agarose gel for separation. RNA was transferred to a positively charged nylon membrane (Zeta-Probe blotting membrane; Bio-Rad) through downward capillary action. The membrane was stained with methylene blue to visualize rRNA and approximate total RNA loading prior to prehybridization and overnight hybridization with a ³²P-radiolabeled riboprobe against the mRNA of interest (ffLuc, rLuc, or VP40). The riboprobes were synthesized using the T7 MaxlScript kit (Ambion) according to the manufacturer's protocol. Five micrograms of template plasmid was linearized overnight and gel purified before the transcription reaction; 50 μ Ci of [³²P]UTP was used in each transcription reaction, and free nucleotides were removed upon reaction completion using Illustra MicroSpin G-25 columns (GE Healthcare). After overnight hybridization, membranes were washed according to the manufacturer's recommendations and exposed to a phosphor screen overnight before being imaged on a Bio-Rad personal molecular imager system. Band intensities of Northern blots and methylene blue staining for total RNA were quantified using Fiji. Band intensities of Northern blots was normalized to RNA loading as measured by intensity of methylene blue staining.

For transfection of total cellular RNA, 2.5 μ g of total RNA was transfected into cells using Lipofectamine 3000 reagent. At 24 h posttransfection, luciferase activity was measured as described above.

Cloning of 3E5E for *in vitro* transcription. The 3E5E-ffLuc sequence was PCR amplified from the NP transcription start site through the ffLuc ORF to the end of the trailer with restriction enzyme sequence overhangs to clone into the pCDNA3 vector in the negative-sense orientation (see Fig. S1 in the supplemental material for the complete vector sequence). The construct was used for expression of the 3E5E-ffLuc mRNA from the T7 promoter directly upstream of the NP transcription start site in an *in vitro* transcription reaction. As a control, the ffLuc ORF was also cloned into the pCDNA3 vector (see Fig. S2 in the supplemental material for the complete vector sequence). *In vitro* transcription of 3E5E-ffLuc and

control fLuc mRNAs was carried out according to the manufacturer's protocol using the mMessage mMachine T7 transcription kit (Ambion) and poly(A) tailing using the poly(A) tailing kit (Ambion).

Immunoblots. Cells were trypsinized and collected in NP-40 buffer (Boston BioProducts) (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, and 5 mM EDTA, pH 7.4 $\pm$ 0.2) supplemented with a cocktail of protease inhibitors (Roche Complete Mini protease inhibitor cocktail). Following cell lysis, nuclear material was removed by centrifugation at 10,000 $\times$ *g* for 10 min at 4°C. Cell lysates were quantified by the Bradford assay (Bio-Rad), analyzed on denaturing Tris-HCl polyacrylamide gels, and transferred onto polyvinylidene difluoride (PVDF) membranes. Proteins of interest were detected by immunoblot analysis using primary antibodies and IRDye secondary antibodies described above and were visualized using a Li-Cor Odyssey CLx (Li-Cor Biosciences). Quantification of immunoblot band intensities was conducted using the LiCor software.

CHX pulse-chase assay. Cells were treated with GC7 for 24 h or not drug treated, transfected with the minigenome components, and then pulsed with either 100 μ g/ml cycloheximide (CHX) or DMSO at 24 h posttransfection for 0, 6, or 12 h. At each time point, a luciferase assay was conducted to assess relative levels of luciferase activity with or without GC7 and with and without CHX. The rate of degradation was normalized to the 0-h time point and then to the DMSO control to compare relative rates of degradation with or without GC7.

[³⁵S]methionine radioactivity assay. Pulse-labeling of BSR-T7/5 cells with [³⁵S]methionine was performed as previously described (29). Cells were treated with drugs for 24 h before washing into medium lacking methionine for 1 h. Cultures were then pulsed with [³⁵S]methionine (12.5 μ Ci/well; PerkinElmer) for 30 min, lysed, and separated by SDS-PAGE. The gel was dried and exposed to a phosphor screen for 24 h before being imaged on a Bio-Rad personal molecular imager system.

Statistics. Statistics were calculated using GraphPad Prism version 6.03 for Windows (GraphPad Software, La Jolla, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01260-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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