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Brief Reports

Creating Higher Titer Lentivirus with Caffeine

Brian L. Ellis,[1] Patrick Ryan Potts,[1] and Matthew H. Porteus[1,2]

Abstract

The use of lentiviral vectors extends from the laboratory, where they are used for basic studies in virology and as gene transfer vectors gene delivery, to the clinic, where clinical trials using these vectors for gene therapy are currently underway. Lentiviral vectors are useful for gene transfer because they have a large cloning capacity and a broad tropism. Although procedures for lentiviral vector production have been standardized, simple methods to create higher titer virus during production would have extensive and important applications for both research and clinical use. Here we present a simple and inexpensive method to increase the titer by 3- to 8-fold for both integration-competent lentivirus and integration-deficient lentivirus. This is achieved during standard lentiviral production by the addition of caffeine to a final concentration of 2–4 m*M*. We find that sodium butyrate, a histone deacetylase inhibitor shown previously to increase viral titer, works only ~50% as well as caffeine. We also show that the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) inhibitor NU7026 can also increase viral titer, but that the combination of caffeine and NU7026 is not more effective than caffeine alone. We show that the time course of caffeine treatment is important in achieving a higher titer virus, and is most effective when caffeine is present from 17 to 41 hr posttransfection. Last, although caffeine increases lentiviral vector titer, it has the opposite effect on the titer of adeno-associated virus type 2 vector. Together, these results provide a novel, simple, and inexpensive way to significantly increase the titer of lentiviral vectors.

Introduction

Lentiviruses are enveloped, positive-sense, single-stranded RNA (ssRNA) viruses of the family Retroviridae. A prototypical lentivirus is the human immunodeficiency virus (HIV). Like other retroviruses, HIV has been modified for use as a vector for gene transfer. An important advantage of lentiviral vectors over other retroviral vectors is their ability to transduce nondividing cells. Although the natural tropism of HIV is T cells and macrophages, recombinant lentiviral vectors can be pseudotyped with the envelope glycoprotein from vesicular stomatitis virus (VSV-G) to allow transduction of a wide variety of cell types, greatly expanding their use (Naldini *et al.*, 1996).

Lentiviruses use the viral integrase protein to catalyze the insertion of the viral genome, which can be engineered to express one or more transgenes, into the host genome. Depending on the multiplicity of infection (MOI), cells can be created to contain one or more independent viral genome integrants. In contrast, integration-deficient lentiviral vectors (IDLVs), which lack viral integrase activity because of a point mutation in the integrase gene, transduce cells but do not efficiently integrate the viral genome into the host genome (Engelman and Craigie, 1992; Kulkosky *et al.*, 1992; Naldini *et al.*, 1996; Vargas *et al.*, 2004; Banasik and McCray, 2010). IDLVs allow transient expression of the gene(s) of interest in dividing cells, which is useful for cell types that are difficult to transfect or transduce by other strategies. More importantly, these vectors could be used in gene therapy protocols to avoid undesired, potentially adverse integrations of a viral genome(s). Retroviral vectors have been used in clinical gene therapy studies for severe combined immunodeficiency (SCID) and chronic granulomatous disease (CGD) gene therapy trials in Europe and the United States (Malech *et al.*, 1997, 2004; Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Gaspar *et al.*, 2004, 2006; Ott *et al.*, 2006; Chinen *et al.*, 2007; Schwarzwaelder *et al.*, 2007). Although the trials were largely successful in treating SCID, random integration of viral DNA led to the development of T cell lymphoblastic leukemia in 4 of 10 patients in the French SCID-XI trial (Hacein-Bey-Abina *et al.*, 2003, 2008; Neschadim *et al.*, 2007). Researchers are now working to create safer gene transfer vectors, the safety of which is now being evaluated in ongoing clinical gene therapy trials (Modlich *et al.*, 2006; Montini *et al.*, 2006; Zychlinski *et al.*, 2008). Lentivirus-mediated transduction of hematopoietic stem cells has been used in gene therapy for

[1]Department of Biochemistry and [2]Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX 75390-9148.

adrenoleukodystrophy (Cartier *et al.*, 2009). In addition, Lombardo and colleagues have shown that IDLV can efficiently deliver zinc finger nucleases (ZFNs) for ZFN-mediated gene targeting in a wide variety of human cell types (Lombardo *et al.*, 2007). These examples demonstrate the broad application of lentiviral vectors for gene transfer. However, the production of high-titer lentiviral vectors, particularly in quantities sufficient for clinical trials, is difficult and expensive. Here, we present a simple and inexpensive method to increase the titer of both integration-competent (ICLV) and integration-deficient lentiviral (IDLV) vectors by 3- to 8-fold.

Materials and Methods

Cell culture

All virus production and cell culture experiments were done in HEK 293FT cells (Invitrogen, Carlsbad, CA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% bovine growth serum (HyClone/Thermo Scientific, Logan, UT), 2 m*M* L-glutamine, penicillin (100 IU/ml), and streptomycin (100 mg/ml). The cultures were grown in a humidified incubator at 37°C with 5% CO_2.

Lentiviral plasmids

The lentiviral vector #277.pCCLsin.cPT.hPGK.eGFP.Wpre, abbreviated 277-eGFP, was a kind gift from L. Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) that carries an enhanced green fluorescent protein (eGFP)-encoding gene driven by the phosphoglycerate kinase (PGK) promoter. The third-generation helper plasmids pMDLg/pRRE D64VInt (produces an integration-deficient virus), pMD2.VSVG, and pRSV-Rev were also generous gifts from L. Naldini. The third-generation helper plasmid pMDLg/pRRE does not have the D64V mutation and produces a functional integrase gene product. The lentiviral vector pLLU2G (Addgene, Cambridge, MA) expresses eGFP driven by a ubiquitin C (UbC) promoter and contains a CAG enhancer and a woodchuck posttranscriptional regulatory element (WPRE). The lentiviral plasmid LGR7 expresses GFP from the UbC promoter and contains the Flap sequence and WPRE as part of its structure. Although these three plasmid vectors share common features, they were created independently and are not direct derivatives of each other.

Caffeine

Caffeine (Fisher Scientific, Hampton, NH) was dissolved in DMEM to a final concentration of 40 m*M*. Briefly, 3.88 g of caffeine medium was added to 500 ml of DMEM. The medium was supplemented with 2 m*M* L-glutamine, penicillin (100 IU/ml), and streptomycin (100 mg/ml) and stored at 4°C. In all experiments, except when otherwise noted, caffeine was added to the cells at both the time of transfection (hour 0) and 17 hr later (hour 17) when the medium was changed.

Lentiviral vector production

Seven hours pretransfection (hour −7), a total of 4×10^6 293FT cells were plated per 10-cm plate in 10 ml of medium. At hour 0, the cells were transiently transfected with 1 ml of a calcium phosphate precipitation mixture containing pMD2.VSVG (3 μg/ml), a 5-μg/ml concentration of either pMDLg/pRRE (for ICLV experiments) or pMDLg/pRRE D64VInt (for IDLV experiments), pRSV-Rev (2.5 μg/ml), and a 10-μg/ml concentration of 277-eGFP (or LGR7 or pLLU2G for the plasmid-specific experiments). At hour 17, the medium was replaced with 10 ml of fresh medium (or 9 ml plus 1 ml of caffeine). At hour 41, the medium was collected and spun at 3000 rpm for 15 min at 4°C. The medium was then filtered through a 0.45-μm pore size Durapore polyvinylidene difluoride (PVDF) membrane (Steriflips; Millipore, Bedford, MA) to remove cellular debris, and then stored at 4°C until use later that day. The viral preparations were not frozen before infection, except as noted. In sodium butyrate experiments, a 1 *M* stock solution in water was diluted to a final concentration of 1 m*M* at transfection (hour 0) and after medium change at 17 hr. In NU7026 experiments, a 10 m*M* stock solution in dimethyl sulfoxide (DMSO) was diluted to a final concentration of 10 μ*M* at transfection (hour 0) and after medium change at 17 hr. In freeze–thaw experiments, half of each sample was frozen at −80°C for 1 hr and then thawed on ice. The other half of the sample was kept at 4°C until the time of infection later that day. In the time course experiments, one-fourth of the cells were replated and the remaining three-fourths of the cells were analyzed by flow cytometry on days 2, 6, 10, and 14.

Adeno-associated virus type 2 production

A total of 4×10^6 HEK 293FT cells were plated per 10-cm dish and were transfected with a 2-ml/plate concentration of a calcium phosphate mixture containing pHelper (5 μg/ml), pAAV-RC (5 μg/ml), and pAAV-GFP (5 μg/ml). The total volume in each plate was 10 ml, including caffeine, which was added at the time of transfection (hour 0) and when the medium was changed the next day (hour 17). At hour 65, 7.5 ml of medium was aspirated from the plates, and the remaining 2.5 ml was used to remove the cells from the plate with a cell lifter, when necessary. This cell suspension was subjected to two cycles of freeze–thawing in a dry ice–ethanol bath and 37°C water bath. The suspension was then incubated with 100 μl of DNase I (1 mg/ml; Roche, Indianapolis, IN) and 10 μl of RNase (1 mg/ml; Roche) for 30 min in a 37°C water bath and then centrifuged for 15 min at 3000 rpm at 4°C. The supernatant was collected and incubated with 0.5% deoxycholic acid (Sigma-Aldrich, St. Louis, MO) for 30 min in a 37°C water bath. The supernatant was then filtered through a 0.8-μm pore size filter and stored at 4°C until use later that day.

Infection and measurement of GFP-positive cells

A total of 100,000 HEK 293FT cells were split into each well of a 24-well plate, in 500 μl of medium. The cells were infected with 5 μl of virus per well (either integration-competent or -deficient lentivirus, or adeno-associated virus type 2 [AAV2]), performed in triplicate with each viral supernatant ($n = 3$). At 24 hr, 500 μl of medium was added to each well. At 48 hr, the cells were harvested and analyzed for GFP expression with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA). Transduction rates were typically 0.1–20.0% and within the linear range of transduction for this cell type. In general, the value for the best titer of 277-eGFP integration-competent lentivirus was ~12,000 IU/μl, whereas the value for the

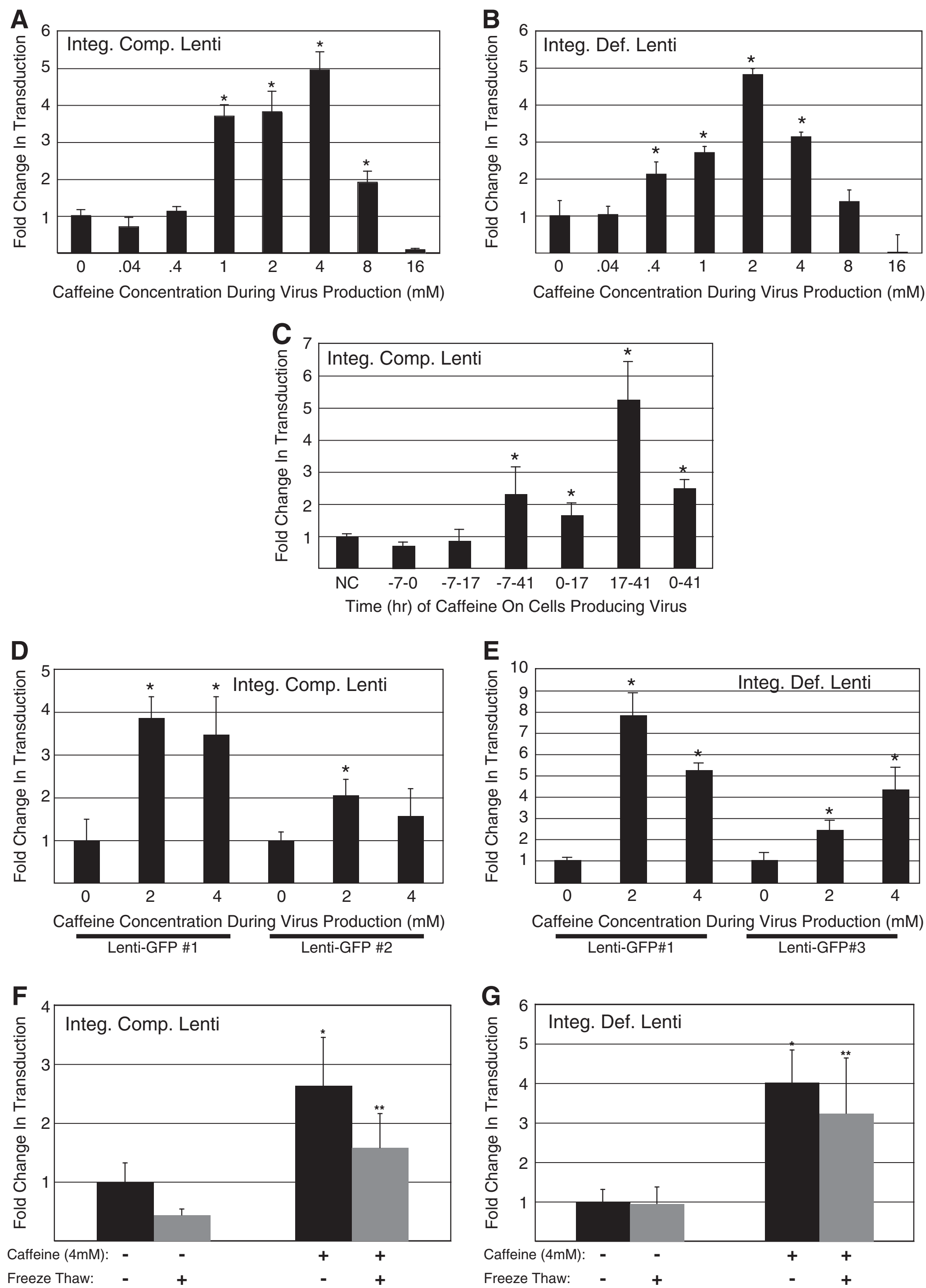


FIG. 1. Effect of caffeine exposure on the titer of lentiviral vectors. (**A**) Fold change in titer of integration-competent lentiviral vector (Integ. Comp. Lenti) 277-eGFP made in the presence of increasing amounts of caffeine (0–16 m*M*). (**B**) Fold change in titer of integration-deficient lentiviral vector (Integ. Def. Lenti) 277-eGFP made in the presence of increasing amounts of caffeine (0–16 m*M*). (**C**) Fold change in titer of integration-competent 277-eGFP made in the presence of 4 m*M* caffeine for various amounts of time. Drug was not added (no caffeine, "NC"), added at the time of split (hour −7), at the time of transfection (hour 0), or at the time of medium change the next day (hour 17). Drug was taken off at transfection (hour 0), at medium change (hour 17), or not taken off and was part of the collection (hour 41). (**D**) Fold change in titer of integration-competent 277-eGFP (Lenti-GFP #1) and integration-competent LGR7 (Lenti-GFP #2) lentivirus made in the presence of 0, 2, or 4 m*M* caffeine. (**E**) Fold change in titer of integration-deficient 277-eGFP (Lenti-GFP #1) and integration-deficient pLLU2G (Lenti-GFP #3) lentivirus made in the presence of 0, 2, or 4 m*M* caffeine. (**F**) Fold change in titer of integration-competent 277-eGFP made in the presence or absence of caffeine and with or without freeze–thawing. (**G**) Fold change in titer of integration-deficient 277-eGFP made in the presence or absence of caffeine and with or without freeze–thawing. *Significantly different compared with the nontreated sample ($n = 3$, $p < 0.05$); **significantly different compared with nontreated plus freeze–thawed sample ($n = 3$, $p < 0.05$).

best titer of 277-eGFP integration-deficient lentivirus was ~6200 IU/μl and was determined according to the following equation: % Gated×total cells infected/volume of virus. The VP/IU ratio, that is, the approximate number of viral particles (VP) per infectious unit (IU), was determined by measuring the amount of p24 in grams (g) and then using the equation: VP = p24 (g)×$1.25e^{16}$.

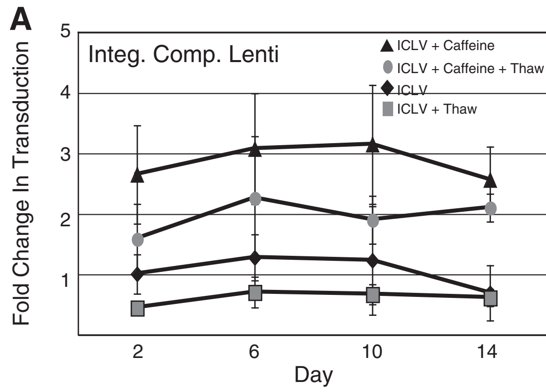
Results and Discussion

The use of lentiviral vectors in both the laboratory and the clinic is broad; however, the production of high-titer virus is difficult and costly. We wanted to find a simple and cost-effective way to increase viral titer by pharmacological means. To this end, we produced GFP-encoding lentiviral particles by standard calcium phosphate transfection of 293FT cells. The supernatant, harvested 48 or 72 hr later, contained newly formed viral particles and was used to infect 293FT cells within the linear range of transduction (typically 0.1–20% transduced cells; data not shown). Two days later the cells were analyzed by flow cytometry for GFP expression and the infectious titer (IU/μl) was determined. We found that adding caffeine during viral production increased the titer of both ICLV and IDLV by 3- to 8-fold (Fig. 1A and B). To determine the optimal caffeine concentration, we added increasing amounts of caffeine during the production of both integration-competent and integration-deficient lentiviral vectors and collected the viral supernatant 48 or 72 hr after transfection. We observed that addition of 2–4 m*M* caffeine for 48 or 72 hr during virus production increased the functional titer of both ICLV and IDLV by 3- to 8-fold (Fig. 1A and B; and data not shown). Addition of greater than 4 m*M* caffeine resulted in significant gross cellular toxicity and did not increase titer (Fig. 1A and B; and data not shown). Next, we performed a time course of cellular exposure to caffeine (4 m*M*) during viral production (Fig. 1C). We found that addition of caffeine from hours 17 to 41 posttransfection resulted in optimal titers (Fig. 1C). Importantly, the effects of caffeine on viral titer are not specific to the 277-eGFP viral backbone, as we found that caffeine had a similar effect on two additional lentiviral backbones (Fig. 1D and E). In addition, the caffeine-induced increase in ICLV and IDLV titer was not significantly affected by freeze–thawing of the virus at −80°C, indicating that the effect of caffeine was not the result of improved cryopreservation (Fig. 1F and G). These results show that addition of caffeine during ICLV or IDLV production increases functional viral titer.

We next examined how caffeine increases functional viral titer. First, we determined whether caffeine increased viral titer by simply raising the transfection efficiency. However, we found that caffeine had no effect on transfection efficiency (data not shown). Another possible explanation could be that caffeine affects viral transduction. However, the concentration of caffeine during transduction in our experiments (~40 μM) has previously been shown not to affect lentiviral transduction (Ariumi *et al.*, 2005). For ICLV, another possible explanation for increased GFP positivity of transduced cells is an increase in nonintegrated proviral forms. However, the increase in GFP positivity is stable over time, indicating that the increase is the result of stably integrated ICLV (Fig. 2A). In contrast, after transduction with IDLV the percentage of GFP-positive cells decreased markedly over time, as expected (Fig. 2B). Importantly, at 14 days posttransduction, when all nonintegrated viral genomes should no longer be present, cells transduced with IDLV made in the presence of caffeine were ~4-fold more GFP positive than cells transduced with untreated IDLV (0.12 vs. 0.03%). These results suggest that caffeine does not affect the number of nonintegrated proviral forms. Last, we examined whether caffeine affects viral particle number by measuring p24 concentration. We found that caffeine had a statistically significant effect on increasing p24 concentration for both ICLV and IDLV (Fig. 2C and D). However, this effect on absolute viral particle number by caffeine does not fully account for its ~4-fold higher functional titer (Fig. 1F and G). This fact is evidenced by the decrease in values of viral particles per infectious unit (VP/IU) on addition of caffeine during the production of both ICLV and IDLV (ICLV – caffeine, 6400 VP/IU; ICLV + caffeine, 3400 VP/IU; IDLV – caffeine, 13,000 VP/IU; ICLV + caffeine, 4400 VP/IU). Taken together, these results suggest that caffeine increases both the number of intact viral particles and the efficiency of packaging the viral particles with viral genomes.

We next examined the cellular target of caffeine. Previous results have shown that inhibition of histone deacetylases (HDACs) by sodium butyrate also increases lentiviral titer (Tang and Taylor, 1990; Yeivin *et al.*, 1992; Olsen and Sechelski, 1995). To examine whether caffeine increases lentiviral titer in a similar manner as sodium butyrate, we treated cells with either caffeine or sodium butyrate alone or in combination. We found that caffeine had a greater effect on

FIG. 2. Mechanistic analysis of caffeine-mediated increased lentiviral vector titers. (**A**) Time course of fold change in titer of integration-competent 277-eGFP made in the presence or absence of caffeine and with or without freeze–thawing. (**B**) Time course of fold change in titer of integration-deficient 277-eGFP made in the presence or absence of caffeine and with or without freeze–thawing. (**C**) Fold change in concentration of p24 (ng/ml) of integration-competent 277-eGFP made in the presence or absence of caffeine and with or without freeze–thawing. (**D**) Fold change in concentration of p24 (ng/ml) of integration-deficient 277-eGFP made in the presence or absence of caffeine and with or without freeze–thawing. (**E**) Fold change in titer of integration-competent 277-eGFP made in the presence of 4 m*M* caffeine (column 2), 1 m*M* sodium butyrate (NaBu, column 3), and 4 m*M* caffeine and 1 m*M* sodium butyrate (column 4). (**F**) Fold change in titer of integration-deficient 277-eGFP made in the presence of 4 m*M* caffeine (column 2), 1 m*M* sodium butyrate (column 3), and 4 m*M* caffeine and 1 m*M* sodium butyrate (column 4). (**G**) Fold change in titer of integration-competent (277-eGFP made in the presence of 4 m*M* caffeine (column 2), 10 μM NU7026 (column 3), and 4 m*M* caffeine and 10 μM NU7026 (column 4). (**H**) Fold change in titer of integration-deficient (277-eGFP made in the presence of 4 m*M* caffeine (column 2), 10 μM NU7026 (column 3), and 4 m*M* caffeine and 10 μM NU7026 (column 4). *Significantly different compared with the nontreated sample ($n = 3$, $p < 0.05$); **significantly different compared with nontreated plus freeze–thawed sample ($n = 3$, $p < 0.05$).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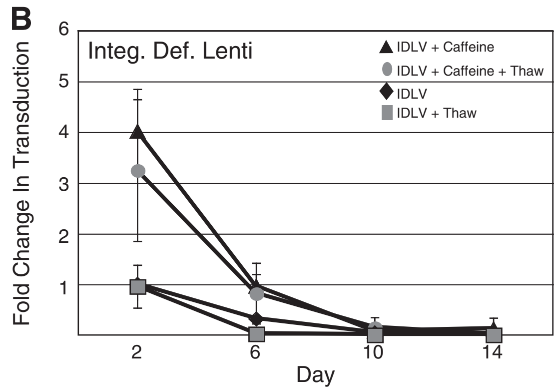
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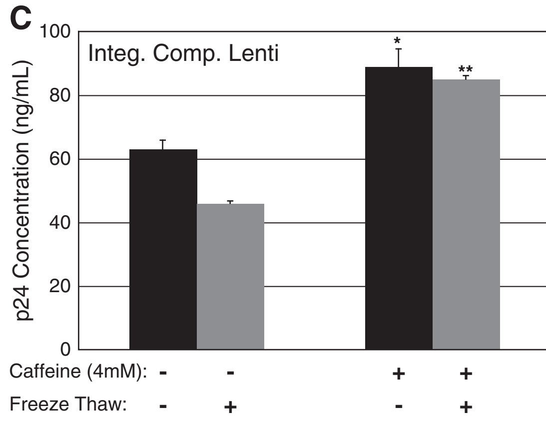
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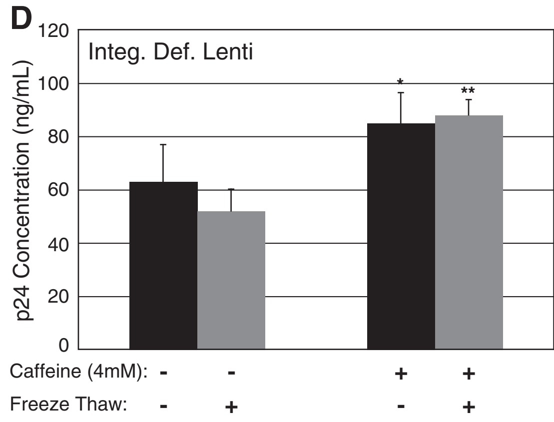
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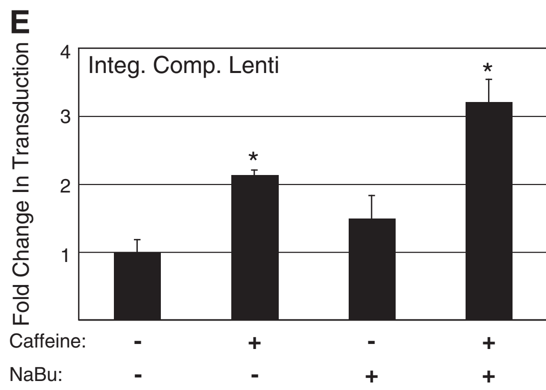
Freeze Thaw: - + - +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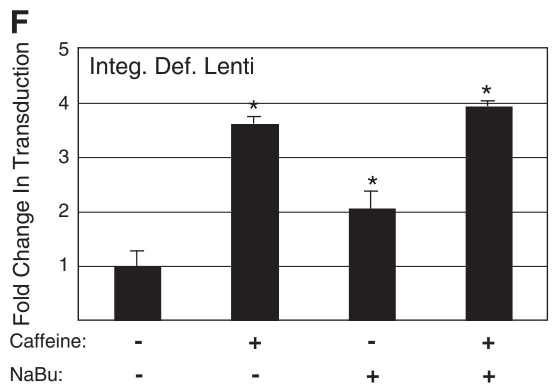
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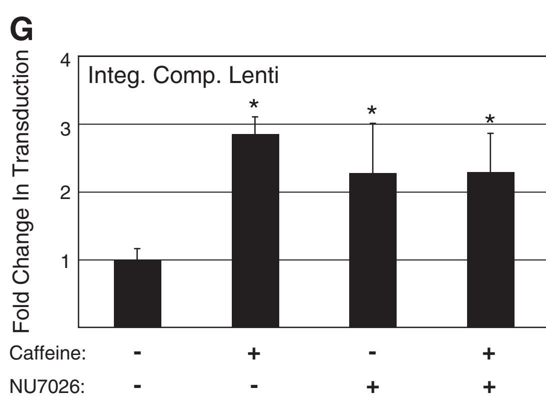
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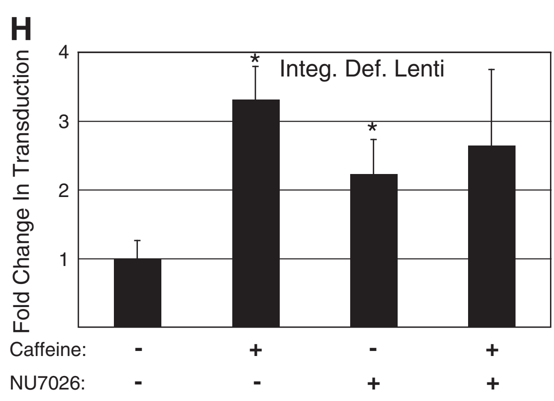
NU7026: - - + +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NU7026: - - + +

increasing viral titer than sodium butyrate when each drug was used alone (Fig. 2E and F). Furthermore, the combination of caffeine and sodium butyrate was more effective than caffeine alone for ICLV, but not IDLV (Fig. 2E and F). Therefore, at least in the case of ICLV, caffeine is unlikely to work via a similar mechanism as sodium butyrate to increase lentiviral titer. Caffeine is a well-established inhibitor of several kinases, including ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which are important signaling proteins involved in the repair of DNA double-stranded breaks (Hall-Jackson *et al.*, 1999; Sarkaria *et al.*, 1999; Block *et al.*, 2004). Therefore, we examined whether specific pharmacological targeting of one of these caffeine targets, DNA-PKcs, would also increase lentiviral titers. Similar to caffeine, we found that treatment of cells with NU7026, a specific DNA-PKcs inhibitor (Veuger *et al.*, 2003), during viral production resulted in higher titers for both ICLV and IDLV (Fig. 2G and H). Furthermore, the combination of caffeine and NU7026 did not increase titer as compared with caffeine alone (Fig. 2G and H). These results suggest that DNA-PKcs is one cellular target of caffeine whose inhibition results in increased lentiviral titer. However, the smaller increase in lentiviral titer induced by NU7026, relative to caffeine, suggests that inhibition of other proteins in addition to DNA-PKcs, such as ATM and ATR, is important in maximizing lentiviral titer(s).

To explore whether the effect of caffeine on lentiviral titer could be expanded to other types of viruses, we tested whether caffeine could increase the titer of AAV2, a parvovirus used frequently as a viral vector for gene transfer. We found that caffeine caused a dramatic decrease in AAV2 vector titer (Fig. 3), indicating that despite having a positive effect on lentiviral titers, caffeine has a negative effect on AAV2 vector titer. The mechanism(s) behind these differences are currently unknown and will be an interesting area for future study.

The increase in lentiviral titer by small-molecule inhibitors such as caffeine and NU7026 suggests that the inhibition of specific proteins enhances lentiviral production. Both caffeine and NU7026 inhibit DNA-PKcs, suggesting that inhibition of a specific gene product, namely DNA-PKcs, is at least partially responsible for the increase in titer achieved with caffeine. However, because caffeine has multiple targets, such as DNA-PKcs, ATM, and ATR, it is likely that inhibition of other caffeine targets may also increase viral titer. In the future, it may be possible to use small interfering RNA (siRNA) knockdowns to identify specific gene targets or pathways, which can also be inhibited to increase lentiviral titer. Once identified, cell-engineering methods, such as ZFN-mediated knockout, would allow for the creation of a higher titer lentiviral production cell line. In addition, although caffeine did not increase the titer of AAV, it may be possible to identify other small molecules that increase the titer of AAV or other viral vectors. If such small molecules were identified then a similar strategy of cell engineering could result in virus-specific higher titer production cell lines. One candidate for this approach is the cellular protein APOBEC3A, which inhibits parvovirus replication (including AAV) (Chen *et al.*, 2006; Narvaiza *et al.*, 2009).

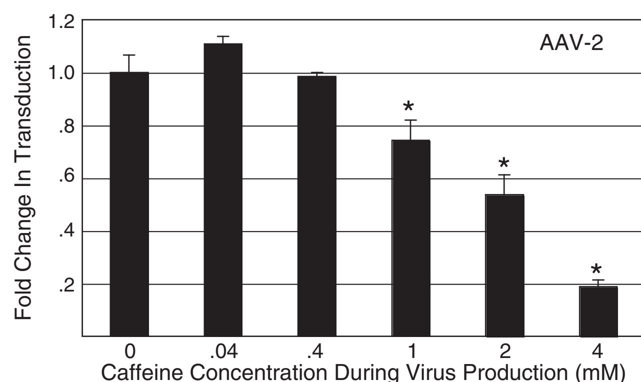

FIG. 3. Caffeine does not increase AAV2 titers. Fold change in titer of AAV2-GFP made in the presence of increasing amounts of caffeine (0–4 m*M*). *Significantly different compared with the nontreated sample ($n = 3$, $p < 0.05$).

In conclusion, we have shown that the addition of 2–4 m*M* caffeine during production of integration-competent or integration-deficient lentivirus can increase the titer up to 8-fold. This method is an easy and inexpensive way to increase the titer of lentiviral vectors and should significantly decrease the cost of lentiviral production for both research and clinical uses.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Matthew H. Porteus
Department of Biochemistry and Department of Pediatrics
University of Texas Southwestern Medical Center
Dallas, TX 75390-9148

E-mail: matthew.porteus@utsouthwestern.edu

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