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**Chemical Stabilization of the HIV-1 Capsid Results in Efficient HIV-1 Reverse
Transcription *in vitro***

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Running Title: Capsid-dependent HIV-1 reverse transcription *in vitro*

Keywords: HIV-1, reverse transcription, capsid, uncoating, inositol hexakisphosphate

23 **ABSTRACT**

24 A defining activity of retroviruses is reverse transcription, the process during which the
25 viral genomic RNA is converted into the double strand DNA required for virus
26 replication. Reverse transcriptase (RT), the viral enzyme responsible for this process,
27 was identified in 1970 by assaying permeabilized retrovirus particles for DNA synthesis
28 *in vitro*. Such reactions are inefficient with only a small fraction of viral genomes being
29 converted to full-length double strand DNA molecules, possibly owing to disruption of
30 the structure of the viral core. Here we show that reverse transcription in purified HIV-1
31 cores is enhanced by the addition of the capsid-binding host cell metabolite inositol
32 hexakisphosphate (IP6). IP6 potently enhanced full-length minus strand synthesis, as
33 did hexacarboxybenzene (HCB) which also stabilizes the HIV-1 capsid. Both IP6 and
34 HCB stabilized the association of the viral CA and RT proteins with HIV-1 cores. In
35 contrast to the wild type, cores isolated from mutant HIV-1 particles containing
36 intrinsically hyperstable capsids exhibited efficient reverse transcription in the absence
37 of IP6, further indicating that the compound promotes reverse transcription by stabilizing
38 the viral capsid. Our results show that stabilization of the HIV-1 capsid permits efficient
39 reverse transcription in HIV-1 cores, providing a sensitive experimental system for
40 analyzing the functions of viral and host cell molecules and the role of capsid
41 disassembly (uncoating) in the process.

42

43 **IMPORTANCE** HIV-1 infection requires reverse transcription of the viral genome.

44 While much is known about the biochemistry of reverse transcription from simplified
45 biochemical reactions, reverse transcription during infection takes place within a viral

46 core. However, endogenous reverse transcription reactions using permeabilized virions
47 or purified viral cores have been inefficient. Using viral cores purified from infectious
48 HIV-1 particles, we show that efficient reverse transcription is achieved *in vitro* by
49 addition of the capsid-stabilizing metabolite inositol hexakisphosphate. Enhancement of
50 reverse transcription was linked to the capsid-stabilizing effect of the compound,
51 consistent with the known requirement for an intact or semi-intact viral capsid for HIV-1
52 infection. Our results establish a biologically relevant system for dissecting the function
53 of the viral capsid and its disassembly during reverse transcription. The system may
54 also prove useful for mechanistic studies of emerging capsid-targeting antiviral drugs.
55

56 INTRODUCTION

57

58 During retrovirus infection, the viral membrane fuses with the target cell, releasing the
59 viral core into the cytoplasm. The core, consisting of a capsid shell surrounding the viral
60 genome and its associated proteins, represents the functional viral payload. In the cell,
61 the viral RNA genome is converted into a double strand DNA molecule by reverse
62 transcription, producing the cis-acting viral sequences necessary for integration and
63 subsequent gene expression. Reverse transcription is catalyzed by the viral reverse
64 transcriptase enzyme (RT) and takes place in a ribonucleoprotein complex housed
65 within the viral capsid. For HIV-1, pharmacological or genetic perturbations of the
66 stability of the capsid typically result in impaired infectivity. Specifically, destabilization
67 of the viral capsid leads to inefficient reverse transcription in target cells (1, 2) while
68 hyperstabilization of the capsid inhibits nuclear entry and integration (3, 4). Similarly,
69 premature capsid disruption in cells expressing restrictive TRIM5 proteins is associated
70 with impaired reverse transcription (5, 6). Collectively, these studies have established
71 that the integrity of the viral capsid is important for efficient HIV-1 reverse transcription
72 in target cells.

73

74 HIV-1 reverse transcription occurs in a series of stages (reviewed in (7)).

75 Synthesis of the minus strand is primed near the 5' end of the genome by a tRNA,
76 resulting in run-off synthesis of a short DNA molecule, the minus strand strong stop.
77 Subsequently, this product anneals to the 3' end of the genome and is extended,
78 resulting in a ~9 kb minus strand product. Plus-strand synthesis is primed by a small

79 RNA remnant at the beginning of the U3 sequence, resulting in a short product that is
80 then extended after annealing to the 3' end of the minus strand. Subsequently,
81 synthesis of the two viral long terminal repeat sequences (LTRs) is completed, resulting
82 in a preintegration complex (PIC) that catalyzes integration of the nascent viral DNA into
83 the target cell genome. A recent study suggests that nuclear entry precedes the
84 completion of reverse transcription, suggesting that the core/reverse transcription
85 complex responds to a specific nuclear signal or is activated during the process of
86 nuclear entry (8). Nonetheless, it is known that active PICs containing two complete
87 DNA ends can be recovered from the cytoplasm of acutely infected cells (9).

88
89 Inositol phosphates are abundant cellular metabolites that participate in a wide
90 array of cell activities (reviewed in (10)). These highly charged small molecules include
91 inositol (1,3,4,5,6) pentakisphosphate (IP5) and inositol hexakisphosphate (IP6). IP6
92 binds to numerous host cell proteins and regulates diverse biological processes,
93 including chromatin remodeling (11), mRNA nuclear export (12, 13), platelet
94 aggregation (14), prion propagation (15), and circadian rhythm (16). IP6 binds to the
95 HIV-1 capsid *in vitro* and stabilizes the hexameric CA lattice. It associates with the
96 center of the CA hexamer, forming ionic interactions with the six Arg18 side chains
97 residing within the hexamer pore formed by the CA N-terminal domains (17, 18). In
98 endogenous reverse transcription reactions with purified HIV-1 cores, the addition of IP6
99 protected the newly synthesized viral DNA from degradation by exogenously added
100 DNaseI *in vitro*, suggesting that the viral capsid can provide an barrier to access of the
101 viral genome (18). IP6 is incorporated into budding HIV-1 particles via an interaction

102 with a distinct site in the assembling Gag lattice, near the CA-SP1 junction (17). It has
103 been proposed that during maturation, IP6 is released upon proteolytic cleavage of Gag
104 and subsequently associates with the mature capsid lattice and stabilizes it (17, 18).
105 The critical importance of capsid stability in HIV-1 reverse transcription and infection,
106 coupled with the relative biochemical instability of purified HIV-1 cores, makes this an
107 appealing model. However, a role of IP6 in reverse transcription itself has not been
108 established.

109

110 Although HIV-1 reverse transcription occurs efficiently in permissive target cells,
111 *in vitro* assays of reverse transcription in permeabilized virions are typically inefficient,
112 for unclear reasons. In these “endogenous reverse transcription” reactions, only a small
113 fraction of viral genomes is converted into full-length double-strand DNA molecules.
114 Such reactions have frequently relied on the addition of detergents or other membrane-
115 disrupting agents to suspensions of concentrated virions, thus permitting access of
116 dNTPs to the viral core (19, 20). The addition of detergents may compromise reverse
117 transcription reactions by destabilizing the viral capsid, resulting in dissociation of RT
118 from the template and its diffusion out of the viral core (21). HIV-1 reverse transcription
119 complexes (RTCs) isolated from acutely infected cells generally lack substantial
120 quantities of the CA protein (22), suggesting that the capsid dissociates during cell
121 permeabilization. The apparent fragility of HIV-1 cores and reverse transcription
122 complexes has hampered biochemical studies of early events in HIV-1 infection,
123 specifically reverse transcription and the role of the viral capsid in this process.

124

125 To address this problem, we identified detergent-free experimental conditions in
126 which HIV-1 cores purified from infectious virions undergo efficient reverse transcription.
127 We show here that addition of the capsid-stabilizing cell metabolite IP6 markedly
128 enhances the efficiency of reverse transcription by promoting the synthesis of full-length
129 minus strand DNA. IP6 also stabilized the association of the CA and RT proteins with
130 HIV-1 cores, suggesting that the effect was mediated by capsid stabilization. Our results
131 are consistent with a model in which the viral capsid promotes retention of a sufficient
132 concentration of RT in association with the viral genome to ensure completion of
133 reverse transcription.

134

135 **RESULTS**

136 **Establishment of the endogenous reverse transcription (ERT) reaction**
137 **using purified HIV-1 cores.** In an effort to improve the efficiency of ERT, we incubated
138 samples of purified HIV-1 cores with dNTPs *in vitro* and analyzed the products by
139 quantitative PCR. For this purpose, we purified HIV-1 cores by a method involving
140 ultracentrifugation of concentrated virions through a layer of Triton X-100 detergent into
141 a sucrose density gradient. Under these conditions, the virions are exposed to the
142 detergent for only a brief time, thus preserving the integrity of the viral core. During
143 centrifugation, HIV-1 cores sediment into the gradient, resulting in removal of the
144 detergent. Cores were detected in gradient fractions by p24 ELISA for the CA protein
145 (Fig. 1A), by assay for RT activity (Fig. 1B), and by negative-stain electron microscopy
146 (Fig. 1C).

147

148 In initial studies, we attempted to improve the reaction efficiency by varying the
149 temperature, reaction time, and pH. We also tested polyethylene glycols which have
150 been previously reported to stimulate reverse transcription *in vitro* (23), and tested the
151 effects of adding bovine serum albumin (BSA). After extended incubation at 37°C, the
152 DNA products were purified and quantified for sequences corresponding to various
153 stages of reverse transcription. An example of the results obtained in this type of
154 experiment is shown in Fig. 2. Quantitative PCR (qPCR) analysis demonstrated that
155 some of these parameters resulted in modest increase in overall ERT efficiency (based
156 on the ratio of 2nd strand transfer products to minus strand strong stop molecules). In
157 particular, the addition of bovine serum albumin appeared beneficial. Nonetheless,
158 based on the quantitative analysis of each stage of reverse transcription, the overall
159 efficiency of the reactions appeared to be limited by a marked attenuation in full-length
160 minus strand synthesis. By contrast, both strand transfer events were relatively
161 efficient. These results indicated that the cores initiated reverse transcription but were
162 unable to synthesize the complete minus strand. Of note, the reactions required
163 prolonged incubation times, suggesting the possibility that the viral capsid dissociated
164 during the incubation, resulting in loss of RT, as previously observed (1).

165
166 **IP6 promotes efficient minus strand synthesis in ERT reactions.** The cell
167 metabolite IP6 was recently reported to bind to the HIV-1 capsid *in vitro* and to promote
168 the assembly of recombinant CA protein into capsid-like structures (17, 18). Adding IP6
169 to permeabilized HIV-1 particles resulted in protection of newly synthesized viral DNA
170 from degradation by DNaseI. By stabilizing the viral capsid, IP6 may prevent access of

171 the nuclease to the nascent viral DNA. However, in that study, addition of IP6 did not
172 enhance ERT when performed in the absence of DNaseI. To test whether capsid
173 stabilization by IP6 can enable efficient ERT, we performed reactions in the presence of
174 a range of IP6 concentrations (Fig. 3). We observed a marked enhancement of full-
175 length minus strand synthesis and increased overall efficiency of the reaction in the
176 presence of low micromolar concentrations of IP6. These results indicate that IP6
177 increases the efficiency of ERT.

178

179 Following this observation, we optimized several parameters in reactions
180 containing 10 μ M IP6, including NaCl and MgCl₂ concentrations and pH. We thus
181 identified conditions for efficient ERT: 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM
182 MgCl₂, 1 mg/ml BSA, 0.5 mM DTT, and 10 μ M IP6, with incubation at 37°C for 16h.
183 Under these conditions, we reproducibly observed conversion of 35 to 40% of the minus
184 strand strong stop products into molecules that also contained HIV-1 sequences that
185 are synthesized only after the second-strand transfer step (Fig. 4). This was the
186 maximum ERT efficiency we observed in multiple experiments with different
187 preparations of cores.

188

189 **Kinetics of the ERT reaction.** We also analyzed the rates of product formation
190 in ERT reactions under the optimized conditions. Minus strand strong stop and 1st
191 strand-transfer products were synthesized rapidly in reactions containing IP6, reaching
192 half-maximal values within 2 h (Fig. 5A). By contrast, synthesis of full-length minus
193 strand and 2nd strand transfer products required 4 to 8 h to reach half-maximal values.

194 In reactions lacking IP6, both early product species were also 50% complete after 2 h,
195 but declined slightly, suggesting the possibility of partial degradation (Fig. 5B). Late
196 products were produced at 2 h in reactions lacking IP6 and declined thereafter. Late
197 products were then slightly increased at the 16h time point, suggesting that degradation
198 may compete with ongoing synthesis. While IP6 stimulated the synthesis of early
199 products up to ten-fold, the effect on late stage reverse transcripts was profound,
200 enhancing product accumulation by several thousand-fold (Fig. 4, compare panels A
201 and B). PCR quantification of the 16h ERT products generated in the absence of IP6
202 using primers spanning the genome revealed that extension of the minus strand was
203 impaired with few DNA products longer than 3 kb accumulating in the reactions (Fig.
204 5C).

205
206 **Hexacarboxybenzene stimulates ERT.** We also tested the synthetic hexavalent
207 compound hexacarboxybenzene (HCB) in ERT reactions, owing to a previous report
208 that HCB stabilizes HIV-1 cores *in vitro* (24). Addition of HCB markedly enhanced
209 minus strand synthesis at an optimal concentration of ~100 μ M, resulting in efficient
210 ERT (Fig. 6A). However, HCB inhibited all stages of ERT when present at a
211 concentration of 1 mM (Fig. 6B), consistent with a previous study reporting inhibition of
212 ERT inhibition by 20 mM HCB (24).

213
214 To determine whether IP6 and HCB stabilize the HIV-1 capsid during ERT, we
215 analyzed the quantity of HIV-1 CA and RT released from viral cores in reactions
216 containing these compounds. Following a 6 h reaction time, the reactions were diluted

217 with cold buffer and subjected to ultracentrifugation to separate the soluble proteins
218 from those which remained core associated. Quantification of the fraction of the total
219 CA present in the pellets revealed higher levels of pelletable CA protein in reactions
220 containing IP6 or HCB, indicating that the viral capsid was stabilized by the compound
221 (Fig. 7). Similarly, assays of RT activity in the supernatants and pellets showed that IP6
222 and HCB increased the levels of pelletable RT in the reactions. Collectively, these
223 results suggest that the enhancing effects of IP6 and HCB on ERT result from
224 stabilization of the viral capsid.

225

226 **Cores from an HIV-1 mutant with a hyperstable capsid undergo efficient**
227 **ERT in the absence of added capsid stabilizers.** We also asked whether genetic
228 stabilization of the HIV-1 capsid affects the dependence of ERT on IP6. For this
229 purpose, we isolated cores from HIV-1 particles containing the capsid-stabilizing CA
230 substitution E45A. This mutant is competent for reverse transcription in target cells but
231 is poorly infectious, owing to impaired nuclear entry and integration (4). Cores from the
232 mutant are hyperstable *in vitro*, as inferred from their increased levels of core-
233 associated CA and slower dissociation of CA during incubation at 37°C (1). In reactions
234 containing IP6, the mutant cores exhibited efficient ERT, as did those from the wild type
235 (Fig. 8). In reactions lacking IP6, E45A cores produced approximately 20% of the late
236 stage products relative to parallel reactions containing IP6. This is in stark contrast to
237 reactions with wild type cores, in which synthesis of late products was less than 0.1% of
238 that observed in reactions containing IP6. These results indicate that E45A mutant
239 cores are capable of synthesizing substantial quantities of late reverse transcripts in the

240 absence of capsid-stabilizing agents, further indicating that the ERT-stimulating activity
241 of IP6 results from stabilization of the viral capsid.

242

243 **The capsid-targeting compound PF74 inhibits ERT in a concentration-**
244 **dependent manner.** Finally, we tested the effects of the capsid-targeting antiviral
245 compound PF74. PF74 binds to a site in the CA hexamer that is distinct from that
246 bound by IP6 (25, 26). When present during HIV-1 infection at concentrations of 10 μM
247 and above, PF74 inhibits reverse transcription and destabilizes the viral capsid (27).
248 Addition of 10 μM PF74 inhibited ERT in reactions containing IP6 (Fig. 9), further linking
249 capsid function to ERT efficiency. By contrast, addition of 1 μM PF74 did not
250 substantially inhibit ERT, consistent with previous reports that at low concentrations
251 PF74 inhibits HIV-1 infection by affecting nuclear entry and integration (28-30). These
252 results further support a role of the viral capsid in ERT.

253

254 **DISCUSSION**

255 In this study, we observed that addition of IP6 stabilizes HIV-1 cores and markedly
256 enhances the efficiency of reverse transcription *in vitro*. Mallery and coworkers had
257 previously shown that IP6 stabilizes purified HIV-1 cores and protects the products of
258 ERT from degradation by added DNaseI, suggesting that the capsid provides a barrier
259 to access to the synthesized DNA (18). In that study, IP6 did not appear to substantially
260 alter the quantity of DNA products in the absence of added DNase. We also observed
261 no enhancing effect of 1 mM IP6 on ERT. By contrast, addition of low concentrations of
262 IP6 resulted in a nearly quantitative conversion of the initial minus strand products to

263 full-length molecules. The observed enhancement resulted from a thousand-fold
264 increase in minus strand synthesis together with modest enhancements at both strand
265 transfer steps. We conclude that IP6 mainly promotes the completion of minus strand
266 synthesis. The maximum efficiency of these reactions was ~40% based on the ratio of
267 2nd strand transfer products relative to minus strand strong stop molecules. Our
268 observations suggest that purified cores can, under appropriate conditions, undergo
269 efficient reverse transcription *in vitro* in the absence of added host proteins.

270

271 IP6 and the synthetic compound HCB also stabilized the association of CA and
272 RT with viral cores, as previously observed in imaging studies of permeabilized virions.
273 Thus, a plausible mechanistic conclusion is that the observed enhancement of ERT
274 resulted from capsid stabilization, because most HIV-1 mutants containing intrinsically
275 unstable capsids are impaired for reverse transcription in target cells (1). We also
276 observed that mutant HIV-1 cores with hyperstable capsids synthesized substantial
277 quantities of full-length minus strand DNA in reactions lacking IP6. Finally, we observed
278 that the capsid-destabilizing HIV-1 inhibitor PF74 inhibited ERT even in the presence of
279 IP6. Together, these observations support a capsid-stabilization mechanism for
280 enhancement of ERT by IP6.

281

282 How might stabilization of the viral capsid help promote the completion of reverse
283 transcription? In our experiments, the addition of IP6 resulted in a profound
284 enhancement of minus strand DNA synthesis with lesser effects on the other steps that
285 were quantified, including both strand transfers. In earlier work, our group showed that

286 spontaneous uncoating of purified HIV-1 cores *in vitro* is characterized by dissociation
287 of both CA and RT from the viral core (1). We suggest that the capsid acts as a
288 container to retain RT during synthesis of the long (~9 kb) minus strand DNA. HIV-1
289 particles are estimated to contain approximately 50 molecules of RT (7), with about 20%
290 of the enzyme copurifying with cores (Fig. 1B). While completion of reverse transcription
291 is theoretically possible with a single molecule of the enzyme, the relatively low
292 processivity of the enzyme together with its frequent pausing suggest that RT must
293 repeatedly rebind the template in order to synthesize full-length viral DNA. By
294 preserving the association of RT with the core, the viral capsid may ensure that RT is
295 maintained at a sufficient concentration to allow completion of the reaction. This
296 “container model” does not exclude additional possible functions of the capsid in
297 reverse transcription, such as providing a scaffold on which the reaction occurs.

298

299 IP6 is a natural metabolite that is present in mammalian cells at concentrations
300 ranging from 40 to 90 μM (31), coinciding well with the ERT-enhancing effects we
301 observed in the present study. Therefore, one could expect that HIV-1 infection would
302 be strongly depending on target cell levels of IP6 and/or the related metabolite IP5.
303 However, a recent study reported that ablation of cell expression of host proteins that
304 synthesize these compounds does not appreciably influence their susceptibility to HIV-1
305 infection (32). These studies were performed in a transformed epithelial cell line, and it
306 will be important to determine whether these inositol phosphates influence HIV-1
307 infection of physiologically relevant target cells. Additionally, purified HIV-1 cores

308 appear to contain substantial quantities of bound IP6 (18), suggesting that the viral
309 capsid may be stabilized by the IP6 it captures during assembly.

310

311 Reverse transcription is thought to promote HIV-1 uncoating in target cells, and it
312 leads to physical changes in HIV-1 cores *in vitro* (33-35). The ERT reaction described
313 herein mimics the kinetics of reverse transcription observed during synchronous HIV-1
314 infection of target cells (36), albeit without the initial lag phase, which presumably
315 results from the requirement for virus fusion. Moreover, the reaction appears to be
316 highly synchronous, with early DNA products appearing rapidly and the subsequent
317 products accumulating only after a substantial delay. The experimental system
318 described herein should enable structural and biochemical studies of native HIV-1
319 reverse transcription complexes and analysis of the role of the viral capsid in the
320 process, including high-resolution analysis of the effects of reverse transcription on the
321 structure of the viral core. ERT reactions with purified cores may also permit the
322 generation of substantial quantities of pure and active HIV-1 PICs *in vitro* for
323 biochemical studies of HIV-1 integration.

324

325 **Materials and Methods**

326

327 **Chemicals, cells, and plasmids.** Inositol hexakisphosphate was purchased as a 1.1M

328 solution from TCI America (cat. No. P0409). D-myo-Inositol-1,3,4,5,6-

329 pentakisphosphate (ammonium salt) was purchased from Cayman Chemical (cat. No.

330 10009851). Tris, mellitic acid, and polyethylene glycol 3350 were purchased from

331 Sigma. Deoxynucleoside triphosphates (dNTPs) were purchased from New England

332 Biolabs as 100 mM solutions. Bovine serum albumin was purchased from RPI (cat. No.

333 A30075). The following antibodies were used for probing immunoblots: 183-H12-5C

334 (NIH AIDS Research and Reference Program (37), used at 4 µg/ml); HIV-1 NC

335 (polyclonal goat serum, from Dr. Robert Gorelick, used at a 1:1000 dilution); HIV-1 RT;

336 HIV-1 IN (polyclonal rabbit serum, received from Dr. Alan Engelman, used at a 1:5000

337 dilution). The IR dye-conjugated polyclonal secondary antibodies were purchased from

338 Li-Cor, Inc. The antiviral compound PF74 was synthesized and purified by the Chemical

339 Synthesis Core of the Vanderbilt University Institute for Chemical Biology. For qPCR,

340 the Maxima SYBR Green/ROX qPCR Master Mix (2X) from ThermoScientific (Cat.

341 #K0233) was used. Custom oligodeoxyribonucleotides were purchased from Integrated

342 DNA Technologies.

343

344 **Purification and analysis of HIV-1 cores.** For most experiments, HIV-1 cores were

345 purified from 200 ml of virus particles collected from infected MT4 cells. Cultured MT4

346 cells (1×10^7) were pelleted and resuspended in 50 ml of medium. Cultures were

347 inoculated wild type HIV-1 particles produced by transfection of 293T cells with the wild

348 type R9 proviral construct. A quantity of the virus stock, corresponding to approximately
349 5 μ g of p24, was added to the MT4 cultures with DEAE-dextran at a final concentration
350 of 20 μ g/ml. The following day, the cells were pelleted and resuspended in 200 ml of
351 fresh culture medium. The cultures were examined daily signs of virus-induced
352 cytopathicity, and at day 4- to 5 after inoculation, the cultures were centrifuged to
353 remove cells and cell debris. The virus-containing culture supernatants were clarified by
354 filtration and concentrated by ultracentrifugation at 32,000 rpm in a Beckman SW32.1Ti
355 rotor at 4°C for 3h. Concentrated virions were resuspended in a total volume of 0.5 ml.

356 For experiments shown in Figure 8, HIV-1 cores were purified from virions
357 produced by transfection of 293T cells, as previously described (38), with the following
358 modifications. Four million 293T cells were transfected with 10 μ g of R9 and R9.E45A
359 plasmid DNAs using polyethyleneimine (39). The next day, cultures were washed and
360 replenished with fresh medium. The following day, the culture supernatants were
361 collected, clarified by filtration through a 0.45 μ m vacuum filtration device, treated with
362 20 μ g/ml DNaseI and 10 mM MgCl₂ at 37°C for one hour to eliminate residual carryover
363 plasmid DNA, then concentrated for purification of viral cores.

364 To purify cores, concentrated HIV-1 particles were subjected to centrifugation
365 through a layer of Triton X-100 (1% vol/vol) into a linear sucrose density gradient, as
366 previously described (38). Fractions (1 ml) were collected and assayed for CA protein
367 by p24 ELISA and RT activity. The dense fractions corresponding to HIV-1 cores
368 typically contained approximately 15% of the total CA protein in the gradients. Fractions
369 containing HIV-1 cores were aliquoted, flash frozen in liquid nitrogen, and stored
370 at -80°C for ERT reactions and other assays.

371 Negative stain electron microscopy of purified cores was performed by applying
372 3-5 μ l samples of core preparations directly to glow-discharged carbon-coated copper
373 EM grids. Following one minute of adherence, the excess liquid was removed by
374 wicking, and the grids were inverted for one minute onto two consecutive droplets
375 containing uranyl formate stain, followed by two consecutive inversions onto water
376 droplets. The liquid was carefully removed by wicking, and the grids were air-dried and
377 imaged in a Morgagni electron microscope. Images were captured using a CCD
378 camera.

379 For immunoblot analysis of HIV-1 cores, 15 μ l volumes of the gradient fractions
380 were separated by electrophoresis on precast 4-20% polyacrylamide gels (Genscript).
381 The proteins were transferred to nitrocellulose membrane, blocked with a solution of
382 nonfat dry milk in TBST, and sequentially probed with antiserum to HIV-1 RT, IN, and
383 CA proteins. Following probing of the blots with the appropriate IR dye-conjugated
384 secondary antiserum, the corresponding HIV-1 protein bands were visualized by
385 scanning the blots in a Li-Cor Odyssey imager.

386
387 **Assays of ERT using purified HIV-1 cores.** ERT reactions were performed in 50 μ l
388 volumes containing 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM
389 dithiothreitol, 0.1 mM each of 4 dNTPs, 1 mg/ml bovine serum albumin, and various
390 concentrations of additives including IP6, mellitic acid, PEG-3350, and PF74. Reactions
391 were normally incubated at 37°C for 16h, after which the DNA was extracted using a
392 silica gel-based method (40) and eluted in water. The products were quantified by
393 qPCR with SYBR green detection using the primers listed in Table 1:

394

395 Table 1. Primers used for quantification of HIV-1 reverse transcripts

Stage of reverse transcription	Primer sequence	Position in provirus sequence
Minus-strand strong stop	5'-GCCTCAATAAAGCTTGCCTTGA-3'	522-543 (F)
	5'-TGACTAAAAGGGTCTGAGGGATCT-3'	592-615 (R)
First strand transfer	5'-GAGCCCTCAGATCCTGCATAT-3'	9493-9513 (F)
	5'-CCACACTGACTAAAAGGGTCTGAG-3'	9682-9705 (R)
Full-length minus strand	5'-CTAGAACGATTGCGAGTTAATCCT-3'	909-932 (F)
	5'-CTATCCTTTGATGCACACAATAGAG-3'	1041-1065 (R)
Second strand transfer	5'-TGTGTGCCCGTCTGTTGTGT-3'	557-576 (F)
	5'-GAGTCCTGCGTCGAGAGAGC-3'	677-696 (R)
Minus strand (8900)	5'-AGGGAAAGAATGAGACGAGC-3'	8835-8854 (F)
	5'-GCTACTTGTGATTGCTCCATG-3'	8904-8924 (R)
Minus strand (7500)	5'-TGGAGTACTGAAGGGTCAAATAAC-3'	7412-7435 (F)
	5'-ACTTCCTGCCACATGTTTATAAATTG-3'	7478-7503 (R)
Minus strand (6000)	5'-TTGTTTCATGACAAAAGCCTTAGG-3'	5937-5960 (F)
	5'-GTCTGACTGTTCTGATGAGCTC-3'	5999-6020 (R)
Minus strand (4500)	5'-GGCAGCTAGATTGTACACATTTAG-3'	4441-4434 (F)
	5'-TGCTGGAATTACTTCTGCTTCT-3'	4481-4502 (R)
Minus strand (2900)	5'-GCAGGGTTAAAACAGAAAAATCAG-3'	2841-2865 (F)
	5'-CCTGAAGTCTTTATCTAAGGGAAGT-3'	2899-2924 (R)
Minus strand (1400)	5'-ACCATGCTAACACAGTGGG-3'	1345-1364 (F)
	5'-AGCTTCCTCATTGATGGTCTC-3'	1396-1416 (R)

396

397 Quantitative PCR reactions were performed in 20 μ l volumes with a Stratagene

398 Mx3000p real time thermal cycler according to the following program: 95° for 10 min (1

399 cycle) followed by 95°C for 30s, 55° for 1 min, and 72° for one min (40 cycles). DNA

400 copy numbers were interpolated from standard curves of C_t values generated from

401 reactions containing dilutions of R9 proviral plasmid DNA, performed in parallel.

402

403 **Uncoating Assays.** ERT reactions (50 μ l) were incubated at 37°C for 6h, diluted with

404 450 μ l cold reaction buffer lacking dNTPs, and subjected to centrifugation at 100,000 $\times g$

405 in a Beckman TLA-55 rotor. Control reactions were diluted and pelleted immediately

406 after mixing. The supernatants were withdrawn and transferred to clean tubes, and the

407 pellets were resuspended in ERT reaction buffer (0.5 ml). The supernatants and pellets

408 were assayed for CA protein by p24 ELISA (41) and for RT activity using an exogenous
409 primer-template assay, as previously described (42). The resulting values were used to
410 calculate the fraction of pelleted CA and RT activity in the samples.

411

412 **Acknowledgments**

413

414 This work was supported by NIH grants R56 AI076121 and P50 AI150481 (Pittsburgh
415 Center for HIV-Protein Interactions). We thank the staff of the Vanderbilt Cryo-Electron
416 Microscopy Facility for training and facilities for electron microscopy and Dr. Greg Sowd
417 for critical reading of the manuscript.

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- 552

553 **FIGURE LEGENDS**

554

555 FIG 1 Characterization of purified HIV-1 cores. HIV-1 cores were isolated from
556 concentrated virions by sucrose density gradient sedimentation. Gradient fractions were
557 assayed for (A) CA protein and (B) RT activity. Panel C shows electron micrographs
558 from negative stained samples of the three gradient fractions containing HIV-1 cores.

559

560 FIG 2 Representative results from an ERT experiment. HIV-1 cores were incubated for
561 16h in a preliminary ERT reaction buffer containing the indicated concentrations of
562 polyethylene glycol 3350. DNA was purified in the reactions and analyzed by qPCR for
563 the indicated products of reverse transcription. This experiment was one of many early
564 attempts to improve the efficiency of ERT.

565

566 FIG 3 IP6 markedly stimulates ERT in vitro by enhancing minus strand synthesis. ERT
567 reactions containing the indicated concentrations of IP6 were incubated at 37°C for 16h,
568 and DNA products were purified and quantified.

569

570 FIG 4 Optimization of the ERT reaction. ERT reactions containing 10 μ M IP6 and the
571 indicated conditions were incubated and analyzed for HIV-1 DNA products. The
572 variables were: (A) NaCl concentration, (B) pH, in reactions containing 150 mM, and (C)
573 MgCl₂ concentration, in reactions at pH 7.6 with 150 mM NaCl.

574

575 FIG 5 Time course of ERT in the presence and absence of IP6. ERT reactions
576 containing and lacking 10 μ M IP6 were incubated for the indicated time periods and
577 subsequently analyzed for various HIV-1 DNA products by qPCR. (A) reactions
578 containing IP6; (B) reactions lacking IP6. Values represent averages of duplicate ERT
579 reactions. (C) Quantification of products in 16h reactions for sequences spanning the
580 viral genome in reactions containing and lacking IP6. The blue and green symbols
581 represent values from pairs of ERT reactions from two different experiments. Dashed
582 lines connect values from ERT reactions lacking IP6. Results shown in this figure are
583 from one of two independent experiments.

584

585 FIG 6 The capsid-stabilizing compound HCB also stimulates ERT. Reactions were
586 containing the indicated concentrations of HCB were incubated for 16h at 37°C, and the
587 products were analyzed by qPCR. Results shown are representative of two
588 independent experiments.

589

590 FIG 7 IP6 and HCB stabilize viral cores during reverse transcription. ERT reactions
591 containing no additive, 10 mM IP6, or 100 mM HCB not incubated or incubated at 37°C
592 for six hours. Reactions were diluted tenfold with reaction buffer, and the cores were
593 pelleted by ultracentrifugation and the pellets and supernatants analyzed for CA and RT
594 activity. Shown is the fraction of the total CA and RT activity in the pellets. The values
595 shown are the average values of duplicate reactions from one of two independent
596 experiments, which showed similar outcomes.

597

598 FIG 8 Cores from the E45A HIV-1 mutant, which contains a hyperstable capsid, are less
599 dependent on IP6 or HCB for ERT. Cores were purified from HIV-1 particles that had
600 been produced by transfection of 293T cells. ERT reactions were performed with and
601 without added IP6 or HCB.

602

603 FIG 9 The capsid-targeting antiviral compound PF74 inhibits ERT. Optimized ERT
604 reactions containing indicated concentrations of PF74 were incubated for 16h.
605 DNA products were purified and analyzed for HIV-1 sequences by qPCR. These results
606 are representative of 3 independent experiments.

607

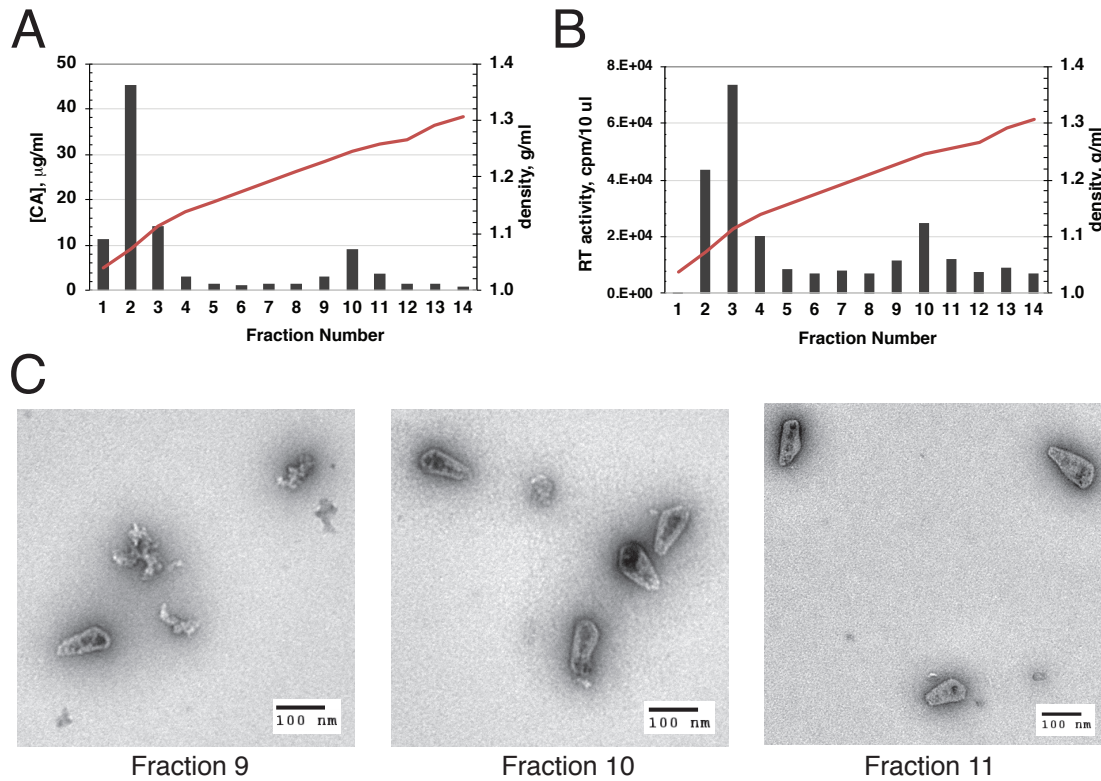


FIG 1 Characterization of purified HIV-1 cores. HIV-1 cores were isolated from concentrated virions by sucrose density gradient sedimentation. Gradient fractions were assayed for (A) CA protein and (B) RT activity. Panel C shows electron micrographs from negative stained samples of the three gradient fractions containing HIV-1 cores.

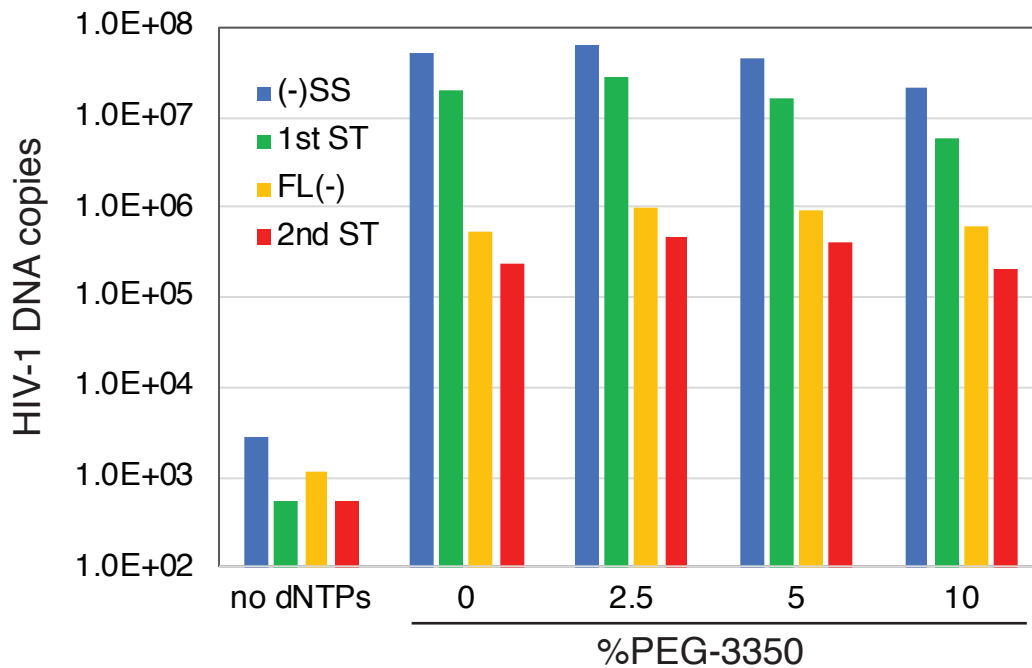


FIG 2 Representative results from an ERT experiment. HIV-1 cores were incubated for 16h in a preliminary ERT reaction buffer containing the indicated concentrations of polyethylene glycol 3350. DNA was purified in the reactions and analyzed by qPCR for the indicated products of reverse transcription. This experiment was one of many early attempts to improve the efficiency of ERT.

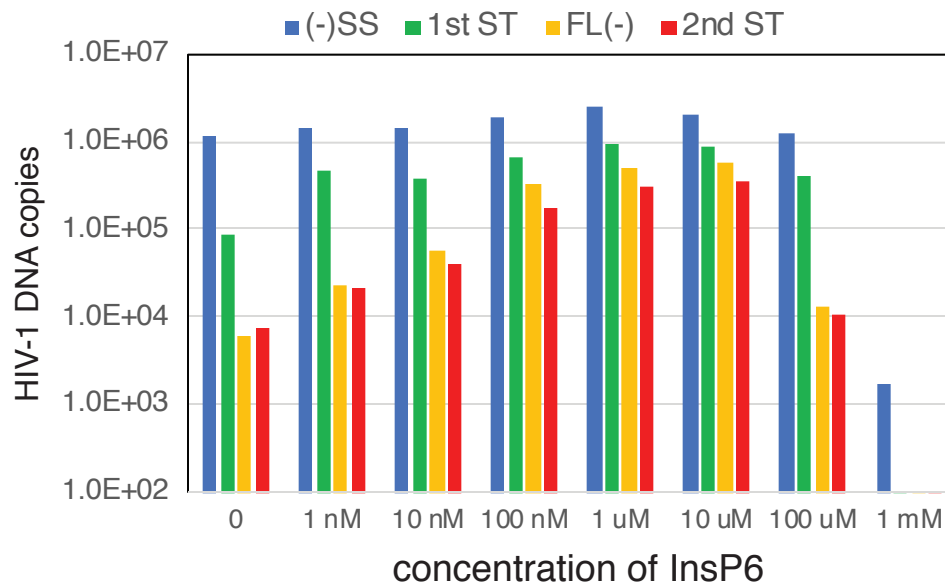


FIG 3 IP6 markedly stimulates ERT in vitro by enhancing minus strand synthesis. ERT reactions containing the indicated concentrations of IP6 were incubated at 37°C for 16h, and DNA products were purified and quantified.

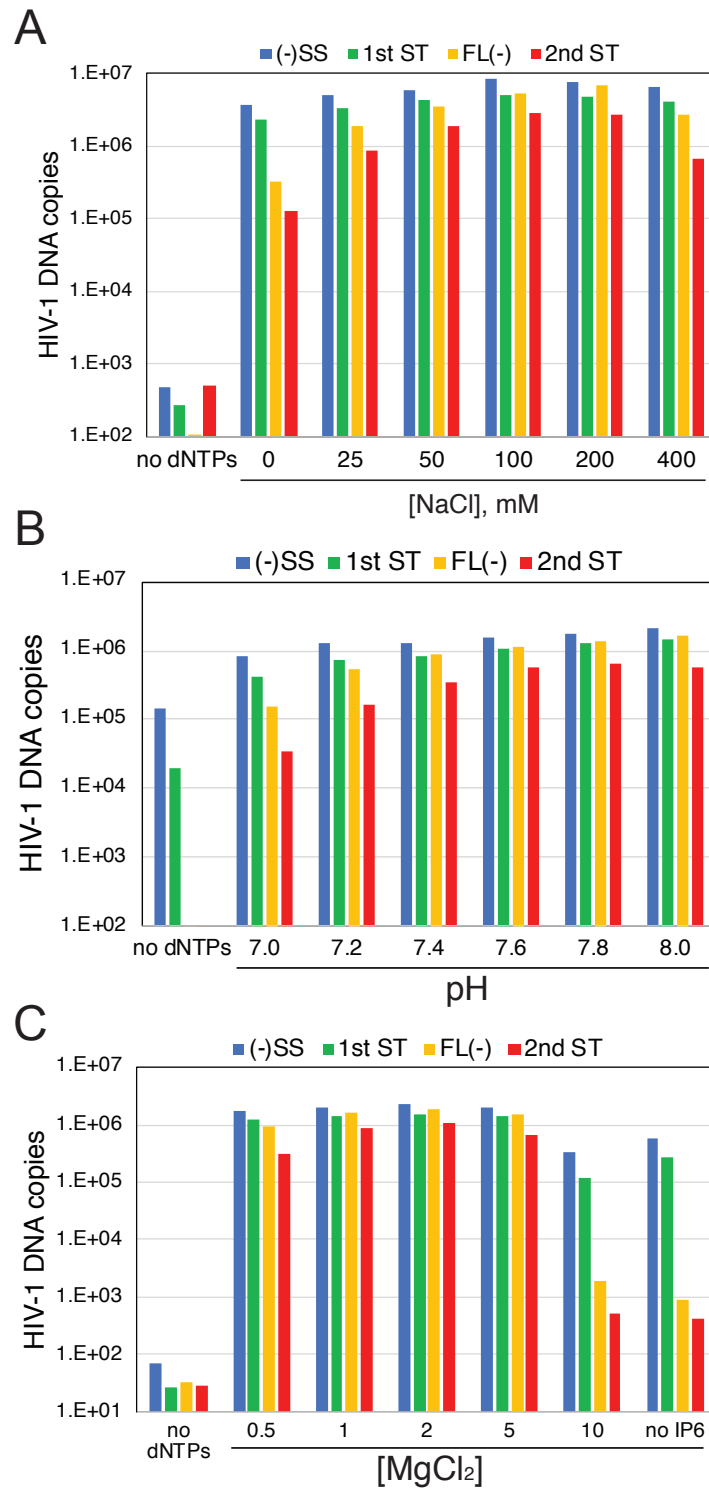


FIG 4 Optimization of the ERT reaction. ERT reactions containing 10 μ M IP6 and the indicated parameters were incubated and analyzed for HIV-1 DNA products. The variables were: (A) NaCl concentration, (B) pH, in reactions containing 150 mM, and (C) MgCl₂ concentration, in reactions at pH 7.6 with 150 mM NaCl.

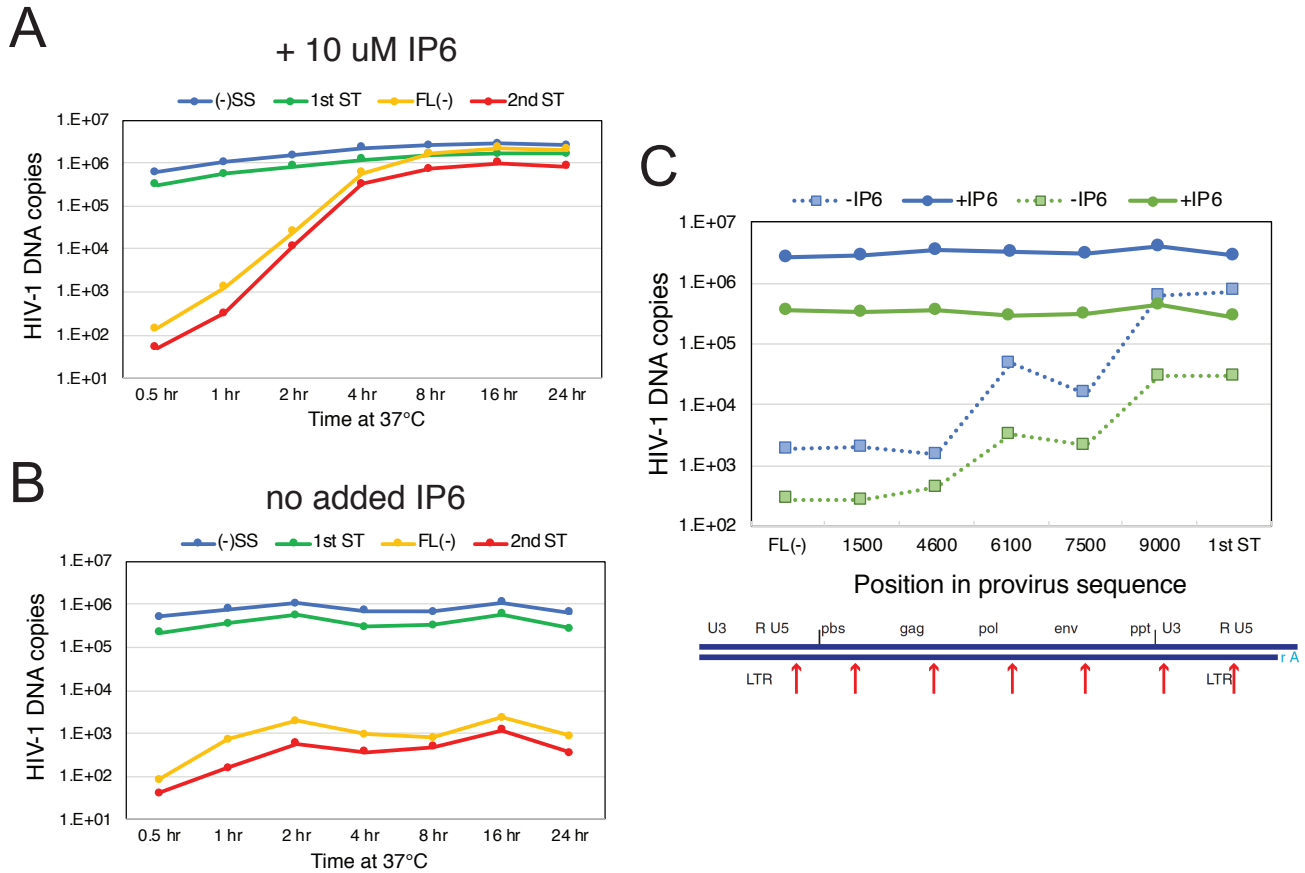


FIG 5 Time course of ERT in the presence and absence of IP6. ERT reactions containing and lacking 10 μ M IP6 were incubated for the indicated time periods and subsequently analyzed for various HIV-1 DNA products by qPCR. (A) reactions containing IP6; (B) reactions lacking IP6. Values represent averages of duplicate ERT reactions. (C) Quantification of products with sequences spanning the viral genome in reactions containing and lacking IP6. The blue and green symbols represent values from independent ERT reactions. Dashed lines connect values from ERT reactions lacking IP6. Results shown in this figure are from one of two independent experiments.

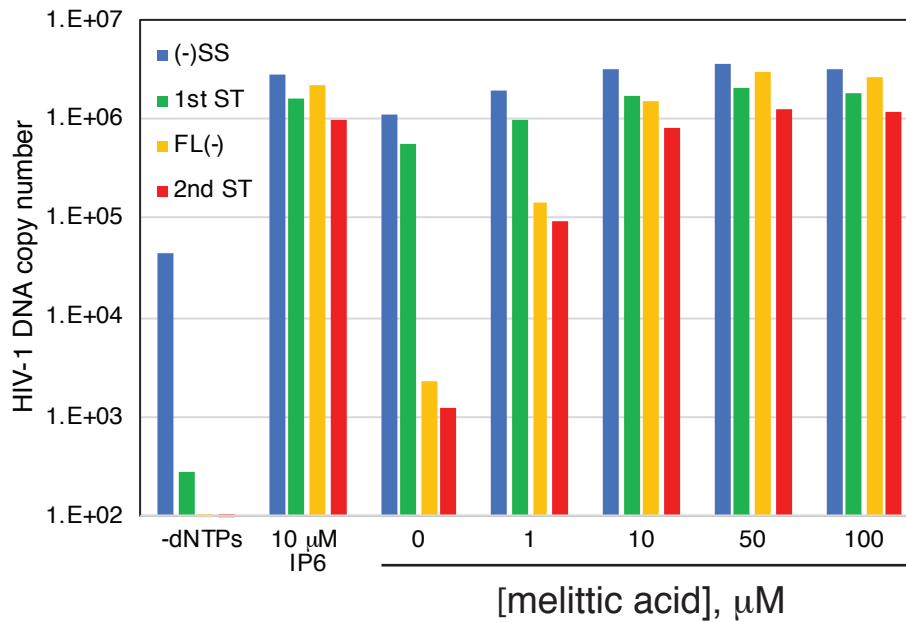


FIG 6 The capsid-stabilizing compound HCB also stimulates ERT. Reactions were containing the indicated concentrations of HCB were incubated for 16h at 37°C, and the products were analyzed by qPCR. Results shown are representative of two independent experiments.

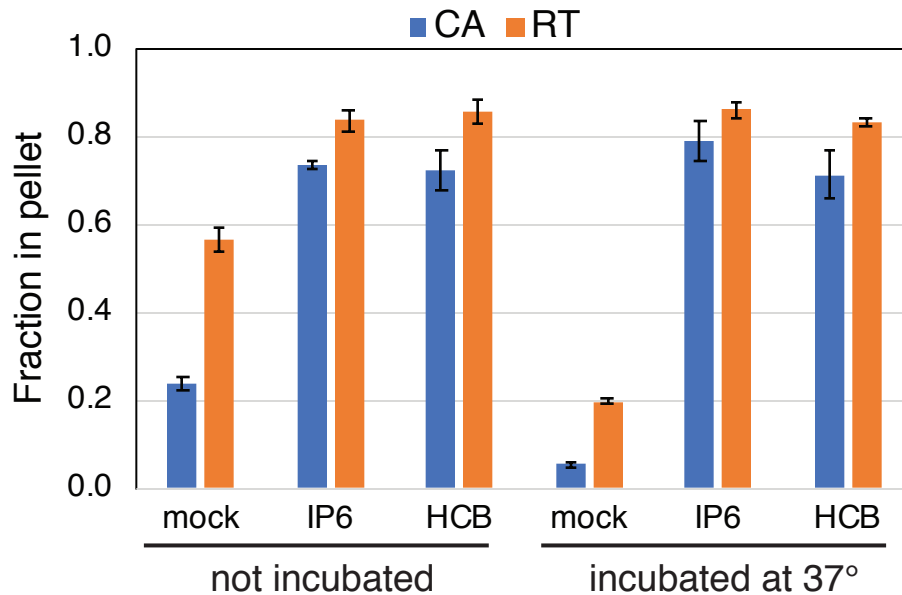


FIG 7 IP6 and HCB stabilize viral cores during reverse transcription. ERT reactions containing no additive, 10 μ M IP6, or 100 μ M HCB not incubated or incubated at 37°C for 6h. Reactions were diluted tenfold with reaction buffer, and the cores were pelleted by ultracentrifugation and the pellets and supernatants analyzed for CA and RT activity. Shown is the fraction of the total CA and RT activity in the pellets. The values shown are the average values of duplicate reactions from one of two independent experiments, which produced similar outcomes.

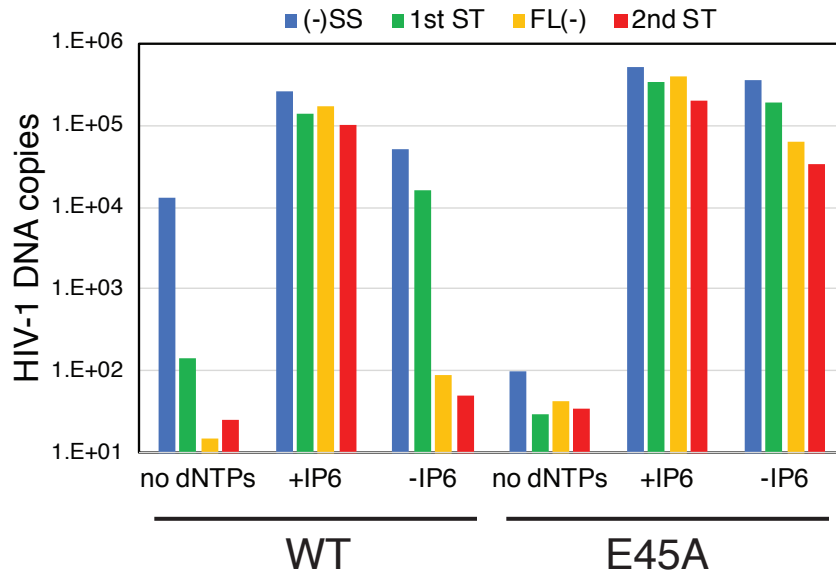


FIG 8 Cores from the E45A HIV-1 mutant, which contains a hyper-stable capsid, are less dependent on IP6 for ERT. WT and E45A mutant HIV-1 cores were assayed for ERT in reactions with and without added IP6 or HCB.

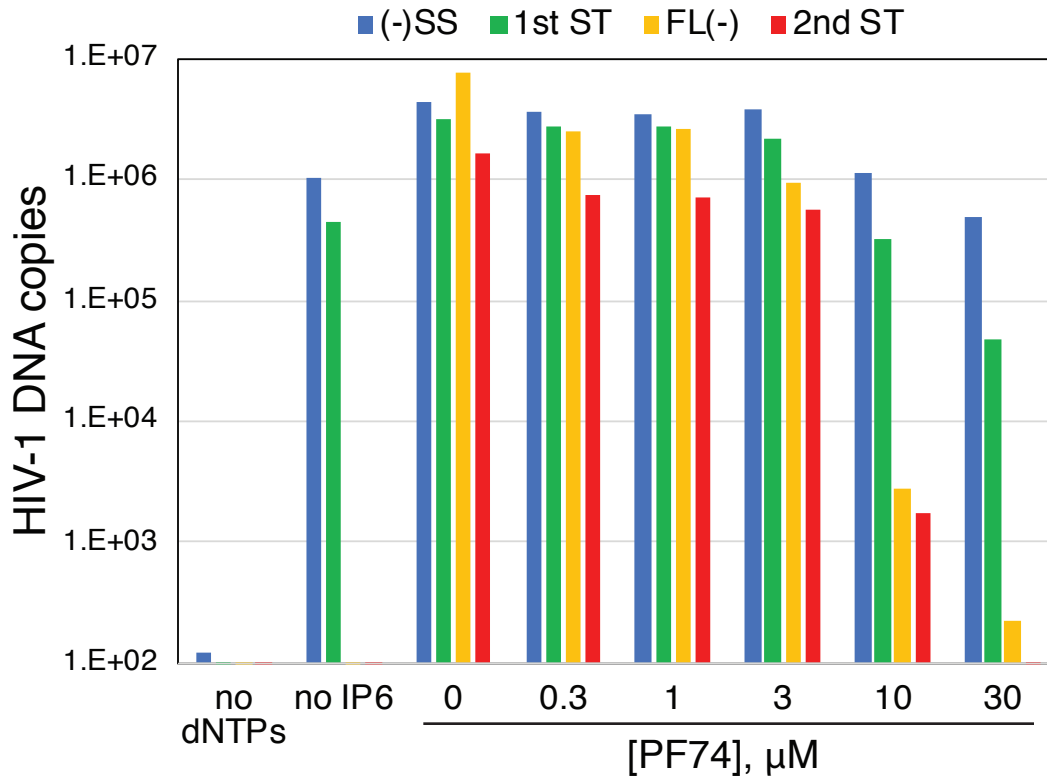


FIG 9 The capsid-targeting antiviral compound PF74 inhibits ERT. Optimized ERT reactions containing the indicated concentrations of PF74 were incubated for 16h. DNA products were purified and analyzed for HIV-1 sequences by qPCR. These results are representative of 2 independent experiments.