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Reduced Viral Replication Capacity of Human Immunodeficiency Virus Type 1 Subtype C Caused by Cytotoxic-T-Lymphocyte Escape Mutations in HLA-B57 Epitopes of Capsid Protein▽

Christian L. Boutwell,[1] Christopher F. Rowley,[1,2,3] and M. Essex[1,2]*

Department of Immunology and Infectious Diseases[1] and Harvard School of Public Health AIDS Initiative,[2] Harvard School of Public Health, 651 Huntington Avenue, Boston, Massachusetts 02115, and Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Lowry Building, Suite GB, 110 Francis Street, Boston, Massachusetts 02215[3]

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Cytotoxic-T-lymphocyte (CTL) escape mutations in human immunodeficiency viruses encode amino acid substitutions in positions that disrupt CTL targeting, thereby increasing virus survival and conferring a relative fitness benefit. However, it is now clear that CTL escape mutations can also confer a fitness cost, and there is increasing evidence to suggest that in some cases, e.g., escape from HLA-B*57/B*5801-restricted responses, the costs to the escape virus may affect the clinical course of infection. To quantify the magnitude of the costs of HLA-B*57/B*5801 escape, a highly sensitive dual-infection assay that uses synonymous nucleotide sequence tags to quantify viral relative replication capacity (RRC) was developed. We then asked whether such CTL escape mutations had an impact equivalent to that seen for a benchmark mutation, the M184V antiretroviral drug resistance mutation of reverse transcriptase (RRC_{V184} = 0.86). To answer the question, the RRCs were quantified for escape mutations in three immunodominant HLA-B*57/B*5801 epitopes in capsid: A146P in IW9 (RRC_{P146} = 0.91), A163G in KF11 (RRC_{G163} = 0.89), and T242N in TW10 (RRC_{N242} = 0.86). Individually, the impact of the escape mutations on RRC was comparable to that of M184V, while coexpression of the mutations resulted in substantial further reductions, with the maximum impact observed for the triple mutant ($RRC_{P146\text{-}G163\text{-}N242}$ = 0.62). By comparison to M184V, the magnitude of the reductions in RRC caused by the escape mutations, particularly when coexpressed, suggests that the costs of escape are sufficient to affect in vivo viral dynamics and may thus play a role in the protective effect associated with HLA-B*57/B*5801.

The capacity of human immunodeficiency virus (HIV) to adapt to, or "escape from," the host cytotoxic-T-lymphocyte (CTL) immune response is well supported by studies of both human infections (31, 45, 46) and experimental infections in the simian immunodeficiency virus (SIV) macaque model (2, 18, 19, 43). CTL escape originates with the introduction, by the error-prone viral reverse transcriptase (RT), of a mutation that disrupts the processing (1, 16), presentation (30, 32, 33), or recognition (11, 48, 51) of a targeted CTL epitope. As a result, the variant viruses expressing the mutation, i.e., CTL escape viruses, increase in frequency over time. However, the ultimate outcome of such CTL escape, both clinically and virologically, is difficult to predict; CTL escape has been associated with loss of virus suppression and disease progression in some cases (7, 10, 20, 26–28, 31, 46) but with continued, or even improved, immune control in others (6, 37). In addition to the potential for clinical impact, vaccine experiments in the SIV macaque model have demonstrated that CTL escape poses a significant threat to the durability and efficacy of CTL-based vaccines (8, 9).

As for any adaptive process, the dynamics of CTL escape, i.e., the direction, rate, and magnitude of change in the population frequency of a CTL escape mutation, are governed predominantly by the impact of the escape mutation on viral relative fitness, a metric that represents the net of all mutation-associated advantages and disadvantages. The reduced CTL destruction of escape virus-infected cells, i.e., increased escape virus survival, confers an increase in relative fitness as evidenced by the outgrowth of escape viruses. However, a number of CTL escape mutations have been identified in both HIV and SIV that appear to carry a concomitant "fitness cost" (21, 22, 33, 36, 37, 42, 44), likely a result of decreased replication capacity of the escape virus (12, 23, 36). The net impact of these opposing fitness effects, i.e., the relative fitness, determines both the intrapatient and interpatient dynamics of CTL escape, and it is therefore important to begin to develop methods to quantify these effects in a meaningful way.

The closely related HLA class I alleles HLA-B*57 and HLA-B*5801 are notable for restricting immunodominant CTL responses that effectively suppress HIV viremia and for being associated with improved long-term clinical outcomes of HIV infection (3, 29, 38, 41). Three of the epitopes that are most strongly targeted by the HLA-B*57/B*5801-restricted CTL response are located in the HIV capsid protein (CA; p24) (3), and CTL escape mutations have been identified for each of them: A146P for IW9 (16), A163G for KF11 (14), and T242N for TW10 (33). The clinical impact of HIV escape from HLA-B*57/B*5801 responses remains unclear; although there are reports of breakthrough viral replication following CTL escape in HLA-B*57/B*5801 epitopes, there are also reports of continued virus suppression despite the presence of CTL escape viruses (6, 7). The latter observation suggests the possibility that escape from HLA-B*57/B*5801 responses may come at a fitness cost that is sufficiently high so as to affect the capacity of

* Corresponding author. Mailing address: 651 Huntington Avenue, FXB 402, Boston, MA 02115. Phone: (617) 432-2334. Fax: (617) 739-8348. E-mail: messex@hsph.harvard.edu.

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the escape virus to replicate to high viral loads. Indeed, the recent descriptions of reduced growth kinetics associated with the T242N mutation in the TW10 epitope (12, 36) and of improved clinical outcomes (lower viral load and higher CD4 count) associated with transmission of viruses expressing the A146P and/or T242N mutations to individuals who do not express HLA-B*57/B*5801 (13) support such a hypothesis.

Here, we describe our efforts to further investigate the impact of HLA-B*57/B*5801 escape mutations on in vivo viral dynamics by quantifying the magnitude of the reductions in the viral relative replication capacities (RRC) of the A146P, A163G, and T242N escape mutations, both individually and, perhaps more physiologically relevant, in combination. Specifically, we address the question of whether the impact of these mutations is sufficient to potentially reduce viral replication in vivo. To do so, we developed a new synonymous-sequence tag dual infection assay that allows direct comparison of the growth of wild-type and variant viruses for the sensitive quantification of RRC and assessed whether the fitness cost for CTL escape mutations is comparable to that seen for a standard drug resistance mutation in the same assay. We provide a benchmark of in vivo relevance by quantifying the RRC of the RT M184V antiretroviral drug resistance mutation, which causes a well-described and clinically important decrease in HIV replication capacity. We then applied the assay to the quantification of the RRC associated with the capsid A146P, A163G, and T242N HLA-B*57/B*5801 escape mutations. In the case of CA T242N, we also assayed the RRC of the alternative CA T242S substitution and of the putative T242N compensatory mutation H219Q (12, 33). The results demonstrate that viral expression of these CTL escape mutations is associated with a fitness cost by virtue of reduced replication capacity, that the cost is cumulative with additional mutations, and that by comparison to RT M184V, the magnitude of the reduction is sufficient to have an impact on in vivo viral loads.

MATERIALS AND METHODS

Molecular clones. The pMJ4 infectious molecular HIV type 1 subtype C (HIV-1C) clone (39) served as the backbone for all viruses used in this study. Several subclones were constructed to provide templates for site-directed PCR mutagenesis. For subcloning convenience, pMJ4 was modified by the deletion of ApaI and BamHI restriction sites from the vector component of the plasmid. Briefly, the ApaI vector site was deleted by partial ApaI restriction digest of pMJ4 followed by fill-in with the Klenow fragment of DNA polymerase I and blunt-end ligation; the resulting plasmid was named pMJ4ΔApa. The BamHI vector site was deleted by restriction digest of pMJ4ΔApa with NotI and EcoRI followed by fill-in with the Klenow fragment of DNA polymerase I and blunt-end ligation to produce pMJ4ΔApaΔBam, which allowed convenient subcloning of fragments containing Nef, RT, and CA.

The *nef* subclone was constructed by restriction digest of pMJ4ΔApaΔBam with BamHI and XhoI and ligation of the 1.5-kb fragment into pCR2.1 (Invitrogen, Carlsbad, CA) to produce pCLB9. The RT subclone was constructed by restriction digest of pMJ4ΔApaΔBam with ApaI and HpaI and ligation of the 1.6-kb fragment into a pCR2.1 vector (Invitrogen, Carlsbad, CA) containing a modified polylinker (XhoI-HpaI/XmaI/SalI/ClaI-ApaI) to produce pCLB11. The subcloning of CA was accomplished in two steps. First, a fragment encompassing the vector, 5′ long terminal repeat, *gag*, and a 5′ portion of *pol* was subcloned by restriction digest of pMJ4ΔApaΔBam with XhoI and ApaI and ligation of the 4.9-kb fragment into pCR2.1 (Invitrogen, Carlsbad, CA) to produce pCLB4. Next, *gag* (and the 5′ portion of *pol*) was subcloned by restriction digest of pCLB4 with XmaI and ApaI and ligation of the 1.5-kb fragment into the same polylinker-modified pCR2.1 vector described above to produce pCLB12.

Cells. HEK293 cells were cultured in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1% (vol/vol) Antibiotic/Antimycotic (Invitrogen) at 37°C with 5% CO_2.

CD8-depleted peripheral blood mononuclear cells (PBMC) were obtained from anonymous HIV-negative blood donors by treatment of whole blood with CD8-specific RosetteSep depletion reagent (Stem Cell Technologies, Vancouver, BC, Canada) followed by gradient centrifugation over Ficoll-Paque (Stem Cell Technologies) per the RosetteSep protocol. Following CD8 depletion, PBMC were washed once in cold 1× phosphate-buffered saline (PBS) supplemented with 1% (vol/vol) FBS, resuspended in a freeze medium consisting of RPMI (Invitrogen) supplemented with 20% (vol/vol) FBS and 10% (vol/vol) dimethyl sulfoxide, and frozen in aliquots at −80°C. Immediately prior to use, PBMC were thawed by gentle incubation in a 37°C water bath and were washed three times in 50 ml PBS supplemented with 1% FBS. Prior to infection, PBMC were stimulated for 3 days in RPMI supplemented with 20% FBS, 1% Antibiotic/Antimycotic, 5 μg/ml phytohemagglutinin (Sigma Aldrich), and 20 U/ml recombinant human interleukin-2 (rhIL-2) (Roche Applied Sciences, Indianapolis, IN) at 37°C with 5% CO_2. After stimulation, PBMC were washed in PBS supplemented with 1% FBS and were cultured in RPMI supplemented with 20% FBS, 1% Antibiotic/Antimycotic, and 20 U/ml rhIL-2 (growth medium) at 37°C with 5% CO_2.

Viruses. Virus stocks were produced by transfection of HEK293 cells with plasmid DNA for full-length infectious molecular clones using Polyfect transfection reagent (Qiagen, Valencia, CA) according to a modified manufacturer protocol. Briefly, at 1 day prior to transfection, 2.8×10^6 HEK 293 cells were seeded in a T75 flask. For the transfection, 15 μg of highly purified plasmid DNA, at a minimum concentration of 1 μg/μl, was diluted to a 150-μl final volume in Dulbecco modified Eagle medium without supplements, 115 μl of Polyfect reagent was added, and the solution was mixed by gentle pipetting and incubated for 10 min at room temperature. During the incubation, medium was removed from the 293 cells to be transfected, they were washed once in cold PBS, and then 7 ml of fresh medium was added. After the 10-min incubation, the transfection mixture was transferred to the flask, swirled gently to mix, and incubated for 3 h at 37°C with 5% CO_2. After 3 hours, the medium was removed and discarded, the cells were washed once with PBS, and 7 ml of fresh medium was added before returning the cells to the incubator. Transfection supernatant was harvested after 72 h, filtered through a 0.2-μm filter, and stored in aliquots at −80°C.

The initial screen of virus stocks was conducted by p24 enzyme-linked immunosorbent assay (Perkin-Elmer, Waltham, MA) per the manufacturer's protocol; stocks that contained less than 10 ng of p24 per ml were discarded. Titers of stocks with a sufficient p24 titer were subsequently determined on CD8-depleted PBMC by 50% tissue culture infective dose per a standard protocol (NIH-NIAID-DAIDS).

***nef* synonymous-sequence tag.** First, the ABI_nef_for_wt (GCAACACAGCC GCCAATAAT) and ABI_nef_reverse (CGCTCCCTTATAAGTCATTGGT CTT) Sybr green quantitative PCR primers were selected using the ABI Prism Primer Design software. Next, the primer binding sites were examined to identify a small number of synonymous nucleotide substitutions in one of the sites that would cause maximal, and approximately equal, destabilization of mismatch binding of both the wild-type primer on the synonymous template and a synonymous primer on the wild-type template but would also result in approximately equal annealing temperatures for each of the primers with its matched template. This resulted in the selection of the synonymous nucleotide substitutions A8940T, C8943G, C8946G, and T8949C, located in the middle of the forward primer binding site, and creation of the matching forward primer, ABI_nef_for_syn (CAACACTGCGGCGAACAAT). Next, the synonymous substitutions were introduced into the pCLB9 MJ4 *nef* subclone by QuikChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis per the manufacturer's protocol with primers as designed by the online Stratagene QuikChange primer design software. The mutagenesis was confirmed by sequencing with Nef4140 (GAGGGCTATCTGCAATATAC) and Nef4436 (GCTCCCTTATAAGTCAT TGG) primers, the BamH I/XhoI fragment was cloned back into pMJ4ΔApaΔBam to produce the full-length infectious pMJ4-*nef*^{SYN}, and the synonymous substitutions were reconfirmed by sequencing the full-length clone with the Nef4140 and Nef4436 primers.

ASPCR. Allele-specific quantitative PCR (ASPCR) was performed on the Applied Biosystems Prism 7500 instrument using the QuantiTECT Sybr green RT-PCR kit (Qiagen, Valencia, CA) per the manufacturer's protocol in a 25-μl reaction volume. Briefly, each reaction mixture consisted of 12.5 μl 2× QuantiTECT Sybr green RT-PCR master mix, 1.25 μl each of 6 μM stocks of allele-specific forward and common reverse primers, 0.25 μl QuantiTECT RT mix, 7.75 μl of distilled water, and 2.0 μl of sample. The cycling conditions consisted of a 30-min RT step at 50°C, a 15-min initial activation and denaturation step at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C, and a melting curve consisting of 1 min at 60°C followed by ramping to 95°C with continuous sampling.

The quantification standards consisted of near-full-length linear plasmid DNA generated by XmaI digest of pMJ4-*nef*^{WT} and pMJ4-*nef*^{SYN} followed by agarose gel purification and spectrophotometric copy number quantification. Independent standard curves were generated for both the *nef*^{WT} and the *nef*^{SYN} allele-specific reactions by quantification of a series of seven 10-fold dilutions of the linearized plasmid standards (5 copies/μl to 5×10^6 copies/μl) quantified in duplicate in each of three independent assays. The cumulative data for each reaction, comprising six data points at each standard dilution, were used to generate a standard curve by regression. The standard curve was calibrated to each subsequent run by including in each run the 5×10^4 standard in duplicate and using the crossing-threshold data to adjust the *y* intercept of the standard curve.

HIV-1C capsid mutants. All capsid escape mutations were considered in the context of the HIV-1C consensus capsid. Recreation of the HIV-1C consensus capsid in MJ4 required the introduction of 10 nucleotide substitutions (C1225G, G1277C, A1446C, G1532C, C1543T, A1544C, A1555G, T1663C, T2619G, and G2620C [HXB2 numbering]) by successive rounds of QuikChange II (Stratagene, La Jolla, CA) site-directed mutagenesis of the pCLB12 Gag subclone per the manufacturer's protocol using primer sets as designed by the online Stratagene QuikChange primer design software. Successful mutagenesis was confirmed following each round by sequencing with Gag551 (CAAGGGCAAATG GTACAC) and Gag984 (ATGCTGACAGGGCTATAC) primers. Finally, the CA CONS C fragment was cloned into the pMJ4-*nef*^{WT} and pMJ4-*nef*^{SYN} backbones (via the pCLB4 intermediate) to produce pMJ4-CA CONS C-*nef*^{WT} and pMJ4-CA CONS C-*nef*^{SYN}, and the CA mutations and *nef* tags were confirmed by sequencing the full-length clones with Gag551, Gag984, Nef4140, and Nef4436 primers.

Next, the mutations A146P (G1225C), A163G (C1277G), H219Q (C1446A), T242N (C1514A), and T242S (C1514G) were introduced into the HIV-1C consensus capsid by QuikChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis of the HIV-1C consensus capsid subclone. Double and triple mutants were produced by successive rounds of mutagenesis. All mutagenesis was confirmed by sequencing with Gag551 and Gag984 primers prior to cloning back into the pMJ4-*nef*^{WT} and pMJ4-*nef*^{SYN} backbones (via the pCLB4 intermediate) to produce the full-length *nef* synonymous tagged MJ4 recombinant escape viruses. Full-length clones were confirmed by sequencing with Gag551, Gag984, Nef4140, and Nef4436 primers.

HIV-1C RT M184V mutant. The RT M184V mutation (A3099G [HXB2 numbering]) was introduced into the pCLB11 subclone by QuikChange II (Stratagene, La Jolla, CA) site-directed mutagenesis per the manufacturer's protocol using primers as designed by the Stratagene QuikChange primer design software. Mutagenesis was confirmed by sequencing with Pol622 (TTGTGTCCTAAAG GGCTCTA) and Pol1077 (GGATGGCCCTAAGGTTAAAC) primers prior to cloning into pMJ4-CA CCONS-*nef*^{WT} and pMJ4-CA CCONS-*nef*^{SYN} to produce the pMJ4-RT V184-*nef*^{WT} and pMJ4-RT V184-*nef*^{SYN} viruses. The M184V mutation and *nef* sequence tags were confirmed by sequencing the full-length plasmids using Pol622, Pol1077, Nef4140, and Nef4436 primers.

RRC Assay. For each infection, 2×10^6 CD8-depleted PBMC were seeded per well in a 24-well plate and were stimulated for 72 h in RPMI supplemented with 20% FBS, 1% Antibiotic/Antimycotic, 5 μg/ml phytohemagglutinin (Sigma Aldrich), and 20 U/ml rhIL-2 (Roche Applied Sciences; Indianapolis, IN) at 37°C with 5% CO_2. After 72 h, the stimulation medium was gently removed and was replaced with 2 ml of PBMC growth medium. Each well was inoculated with two viruses: the "wild-type" virus expressing a HIV-1C consensus capsid and one of the *nef* tags and a "mutant" virus expressing the mutation(s) of interest and the other *nef* tag. The total multiplicity of infection (MOI) was 0.001. After overnight incubation, the inoculum was carefully removed, 500 μl was set stored at −80°C as the day 0 or "inoculum" sample, and 2 ml of fresh PBMC growth medium was added. On days 2 to 7, 500 μl of supernatant was sampled with replacement (growth medium) and stored at −80°C.

Viral RNA was isolated from 140 μl of sample supernatant with a QiaAmp viral RNA minikit (Qiagen, Valencia, CA) per the manufacturer's protocol with the exception of the addition of an on-column DNase step. Briefly, the samples were digested with DNase on a column for 15 min at room temperature with 10 μl of DNase (Qiagen, Valencia, CA) diluted in 70 μl Qiagen RDD buffer, followed by a second AW1 wash before the standard QiaAmp protocol was resumed. Viral RNA was eluted in 50 μl DNase/RNase-free H_2O and was stored at −80°C prior to quantification by ASPCR. All time point samples (day 2 to day 7) for a given infection were quantified in a single ASPCR run.

RRC was calculated by the log relative fitness method described by Dykes et al. (17), which is essentially a linear regression of the change in the ratio of mutant to wild-type virus from time point to time point. We limited the time points analyzed by the log relative fitness method to those in the log-linear phase of infection. The log-linear phase was determined by plotting cumulative (wild-type plus mutant) virus quantities versus time on a log scale and selecting the maximum number of data points that could support a linear regression line with an r^2 value of ≥0.99. The mean replication capacity (with 95% confidence interval) was calculated for each mutation over all wells in which it was assessed using SigmaStat 3.1. The statistical significance of the impact of experimental mutations on RRC was determined by comparison of the mean experimental RRC value to the null expectation of wild-type RRC, which is equal to 1.00 by definition.

RESULTS

Quantification of the impact of mutations on the RRC of HIV comprised three steps: dual infection of target cells with "wild-type" and "mutant" viruses, differential quantification of the "wild-type" and "mutant" viruses by *nef* synonymous-tag ASPCR, and calculation of the RRC of the mutant. The dual infection assay provides a powerful technique for quantifying the impact on HIV replication capacity of a mutation of interest, e.g., drug resistance or CTL escape, because it allows direct comparison of viral growth without the nonspecific variation that can affect parallel but independent infections. However, the assay requires the differential quantification of nearly isogenic viruses from a mixed sample. To accomplish this without disrupting any native virus function, we constructed two recombinant HIV-1C backbones, named MJ4-*nef*^{WT} and MJ4-*nef*^{SYN}, that differed by only four synonymous nucleotide substitutions in *nef*: A9258T, C9261G, C9264G, and T9267C (Fig. 1A). These synonymous sequence tags served as artificial "alleles" to allow differential quantification of the two viruses by ASPCR using tag-specific forward primers and a common reverse primer in parallel but independent reactions (Fig. 1B and C). The *nef*^{WT}-specific and the *nef*^{SYN}-specific ASPCRs exhibited linear quantification across a 7-log-unit range of plasmid reference standard concentrations from 5 copies/μl to 5×10^6 copies/μl (Fig. 1D and E, respectively) with nearly identical high reaction efficiencies as indicated by the slope of the *nef*^{WT} and *nef*^{SYN} standard curves (slope = −3.23 and −3.26, respectively). The tag specificity of each reaction was demonstrated by quantification of mixed template samples containing known quantities of the MJ4-*nef*^{WT} and MJ4-*nef*^{SYN} plasmid reference standards; the *nef* synonymous-tag ASPCR accurately quantified the absolute and relative quantities of the two templates across a broad range of sample mixes (Table 1).

The equivalence of the *nef*^{WT} and *nef*^{SYN} tags with respect to virus replication was demonstrated by quantification of the RRC associated with the *nef*^{SYN} tag. For example, a representative dual infection with an inoculum comprising 43% MJ4-*nef*^{WT} and 57% MJ4-*nef*^{SYN} resulted in a 3-log-unit increase of each virus with approximately equal growth kinetics (Fig. 2A) and a cumulative log-linear growth phase from day 2 to day 6 (Fig. 2B), thereby defining the measurement period as day 2 to day 6. The relative frequencies of the two viruses varied slightly during log-linear growth (Fig. 2C), and thus the per-day change in log-transformed ratio of MJ4-*nef*^{SYN} to MJ4-*nef*^{WT} ranged from −0.16 to 0.28, resulting in an RRC of 1.00 for MJ4-*nef*^{SYN} in this experiment. The RRC associated with expression of the *nef*^{SYN} sequence tag was quantified in a total of eight such dual-infection experiments using inoculation with different initial virus ratios ranging from 70% MJ4-*nef*^{WT}/30% MJ4-*nef*^{SYN} to 20% MJ4-*nef*^{WT}/80% MJ4-*nef*^{SYN}, resulting in a mean RRC^{SYN} of 1.00, which was not significantly different from wild type.

Following validation of quantification by ASPCR and dem-

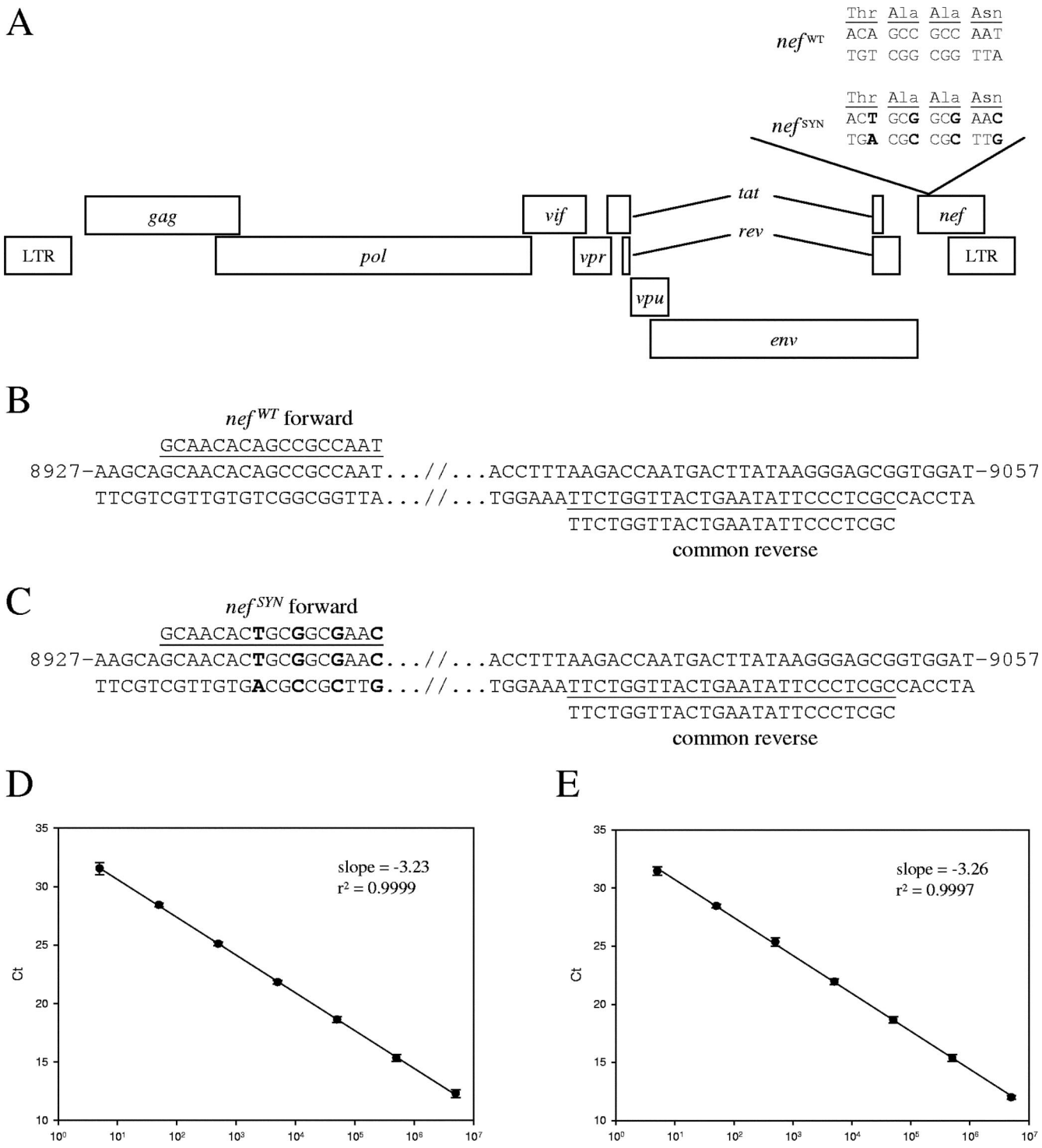

FIG. 1. *nef* synonymous-sequence tag ASPCR. (A) Tagged recombinant HIV-1C backbones were constructed by introduction of four synonymous nucleotide substitutions into the *nef* gene of the MJ4 infectious clone. (B to D) Allele-specific quantitative PCR using a common reverse primer paired with a forward primer specific for the nef^{WT} (B) or nef^{SYN} (C) tag allowed efficient and sensitive quantification over a wide range of nef^{WT} (D)- and nef^{SYN} (E)-tagged template concentrations.

onstration of the equivalence of the two *nef* synonymous-sequence tags, we established a clinically relevant benchmark for the assay by quantifying the RRC associated with the RT antiretroviral drug resistance mutation M184V. Multiple dual-infection assays were conducted at a range of M184/V184 inoculum ratios and with RT V184 expressed in both the MJ4-nef^{WT} and MJ4-nef^{SYN} virus backbones. In contrast to the aforementioned synonymous-tag dual-infection experiments,

TABLE 1. Differential quantification of mixed plasmid template samples by *nef* synonymous ASPCR

Sample composition (%)[b]		*nef* synonymous-tag ASPCR[a]			
		Copies/μl		% of total[c]	
pMJ4-*nef*^{WT}	pMJ4-*nef*^{SYN}	pMJ4-*nef*^{WT}	pMJ4-*nef*^{SYN}	pMJ4-*nef*^{WT}	pMJ4-*nef*^{SYN}
99	1	449,719	4,781	99	1
95	5	446,684	24,475	95	5
80	20	367,034	89,644	80	20
70	30	318,377	139,766	69	31
60	40	307,776	198,032	61	39
50	50	239,559	233,320	51	49
40	60	177,828	258,501	41	59
30	70	137,479	304,565	31	69
20	80	92,195	344,423	21	79
5	95	25,119	440,471	5	95
1	99	4,063	425,677	1	99

[a] *nef* synonymous tagged templates were quantified in parallel *nef* ^{WT}- and *nef*^{SYN}-specific quantitative PCRs.
[b] Mixed samples consisted of known quantities of linearized pMJ4-*nef* ^{WT} and pMJ4-*nef* ^{SYN} plasmid standards.
[c] ASPCR-derived template frequencies in the sample calculated from copy number values.

the RT M184/RT V184 dual infections showed a clear impact of the V184 mutation on viral replication as demonstrated by growth kinetics (Fig. 3A) and changes in relative virus frequency plot over the log-linear growth phase (Fig. 3B) from a representative experiment (initial inoculum of 40% MJ4-RT M184-*nef*^{WT} and 60% MJ4-RT V184-*nef*^{SYN}). Quantification of RRC values in 11 individual dual infections resulted in a mean RRC^{V184} of 0.86, a statistically significant decrease from the defined wild-type RRC of 1 (Fig. 4A).

Similar to the quantification of RRC^{V184}, the impact on RRC of HLA-B57 capsid CTL escape mutations, individually and in combination, was quantified by conducting multiple dual-infection assays across a range of initial mutant/wild-type ratios with the mutation expressed in the MJ4-*nef*^{WT} and MJ4-*nef*^{SYN} virus backbones in an equal number of experiments (all mutations and mutation combinations were analyzed in 10 dual-infection assays except T242N, which was analyzed in 11). The A163G and T242N mutations caused a statistically significant decrease in RRC (mean RRCs = 0.89 and 0.86, respectively), the A146P mutation caused a decrease in RRC (mean RRC^{P146} = 0.91) that was of borderline statistical significance,

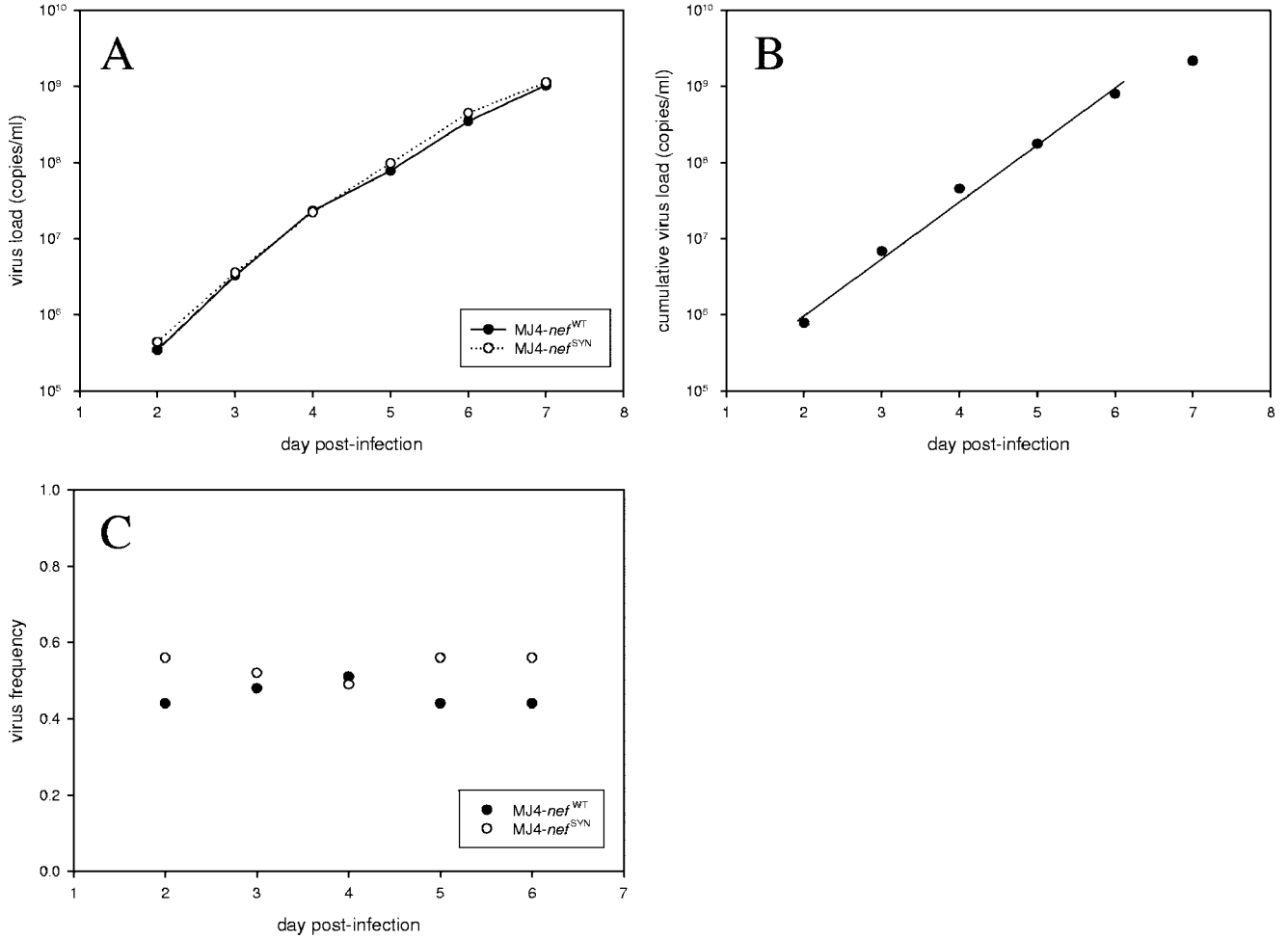

FIG. 2. The *nef*^{SYN} sequence tag does not affect RRC. MJ4-*nef*^{WT} and MJ4-*nef*^{SYN} viruses exhibited similar growth kinetics over a 7-day dual infection (A) with the log-linear growth phase occurring from day 2 to day 6 postinfection (B). The relative frequency of the two viruses varied within a narrow range over the log-linear phase (C). The RRC was quantified by determining the ratio of mutant to wild-type viruses, in this case *nef*^{SYN} to *nef*^{WT} (log transformed), calculating the change in the ratio over each time point, and taking the mean of the values.

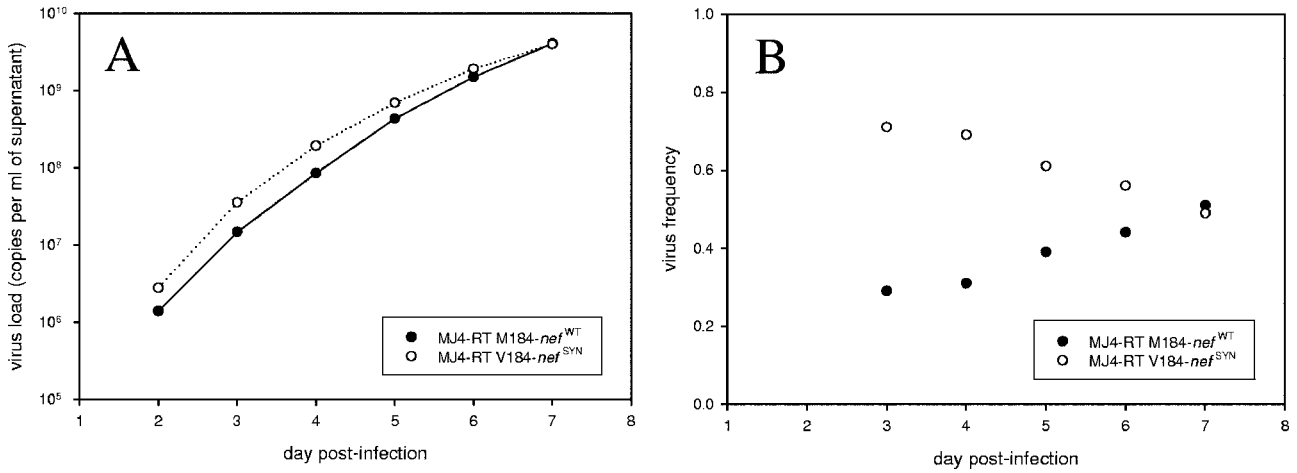

FIG. 3. The RT M184V mutation causes a reduced RRC. The MJ4-RT V184-*nef*^{SYN} virus exhibited reduced growth kinetics relative to the MJ4-RT M184-*nef*^{WT} virus (A), which affected the relative frequencies of the two viruses over time (B).

and the T242S alternative escape mutation did not cause a statistically significant change in RRC (mean $RRC^{S242} = 0.95$) (Fig. 4B). Coexpression of escape mutations caused further reductions in RRC: $RRC^{P146\text{-}G163} = 0.46$, $RRC^{P146\text{-}N242} = 0.73$, $RRC^{G163\text{-}N242} = 0.76$, and $RRC^{P146\text{-}G163\text{-}N242} = 0.62$. All reductions caused by expression of multiple escape mutations were statistically significant (Fig. 4C).

In addition to the three primary HLA-B57 escape mutations in capsid, we also assayed the impact on replication capacity of the H219Q capsid mutation, an apparent compensatory mutation for T242N. Expression of the H219Q mutation individually resulted in a small mean increase in replication capacity relative to wild type ($RRC^{Q219} = 1.08$) which achieved borderline statistical significance, coexpression of H219Q with T242N restored wild-type replication ($RRC^{Q219\text{-}N242} = 0.95$), and coexpression with the alternative T242S mutation had a minimal effect ($RRC^{Q219\text{-}S242} = 0.92$) (Fig. 4D).

DISCUSSION

The adaptation of HIV to the host CTL immune response, manifested as the appearance and increase in population frequency of variant viruses expressing CTL escape mutations, represents a serious challenge to the control of virus replica-

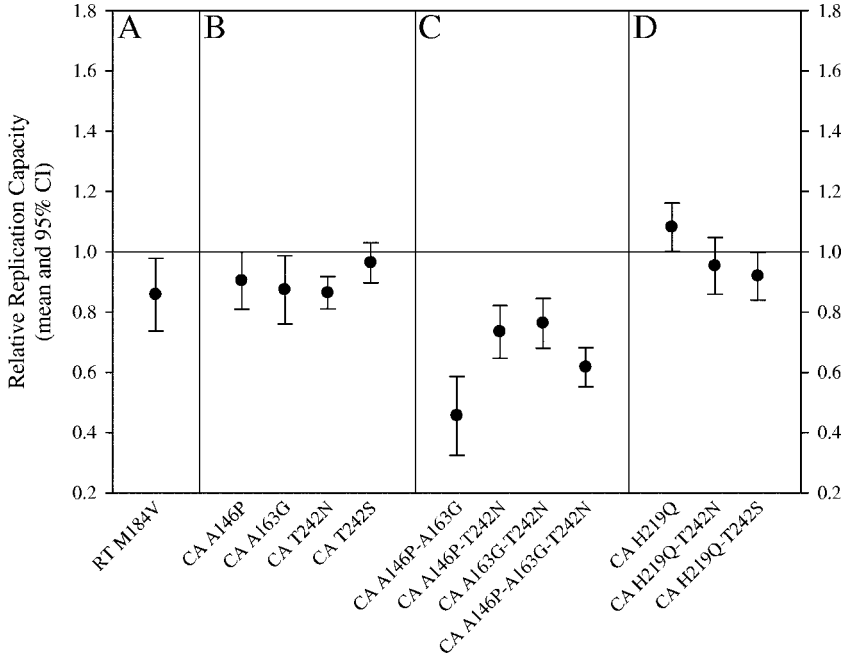

FIG. 4. Quantification of RRC. The mean RRC (dots) with 95% confidence interval (CI) (whiskers) is shown for the benchmark RT M184V mutation (A), the HLA-B57-associated capsid escape mutations expressed individually (B) and in multiples (C), and the putative compensatory mutation CA H219Q individually and with the associated CA T242 mutations (D). The horizontal line denotes the "wild-type" RRC, which is equal to 1.00 by definition.

tion by CTL immune responses, whether induced by infection or vaccination. However, the evidence that CTL escape mutations, like those conferring antiretroviral drug resistance, can impart a viral relative fitness cost (21, 22, 33, 36, 37, 42, 44) raises the possibility that with sufficient understanding of the mechanisms and dynamics of the process, it may be possible to anticipate, mitigate, and perhaps even manipulate CTL escape (4). Indeed, recent reports suggest that CTL escape-associated fitness costs may contribute to the protective effect associated with host expression of particular HLA class I alleles such as HLA-B57 (6, 37). In order to develop sufficient understanding of CTL escape so as to some day co-opt this viral advantage for patient benefit, it is important not only to move from qualitative to quantitative analyses of the replicative cost of CTL escape mutations but also to begin to consider the impact on viral fitness of the coexpression of multiple escape mutations rather than just individual mutations in isolation. The results of this study demonstrate that HLA-B57-associated CTL escape mutations in the HIV-1C capsid cause significant reductions in viral replication capacity when expressed both individually and, as shown for the first time, in combination. We also demonstrate that the magnitude of such CTL escape mutations is equivalent to that seen for the well-known RT M184V antiretroviral drug resistance mutation and thus is sufficiently large so as to have a potential impact on virus replication in vivo.

The restoration of wild-type-level replication capacity by coexpression of the H219Q mutation with the T242N escape mutation supports its previously described role as a compensatory mutation for T242N (12, 33). However, the slight increase in replication capacity associated with individual expression of the H219Q mutation is consistent with prior descriptions of the effect of this mutation on HIV replication (24, 25) and suggests that H219Q may increase replication more generally rather than as a specific compensation for the T242N escape mutation. Indeed, H219Q has also been described to compensate for decreased replication associated with protease inhibitor resistance mutations (40).

The dual-infection assay, defined here as the inoculation of a culture of target cells with both a reference (wild-type) virus and a variant virus, has become a standard method for the in vitro assessment of the impact of adaptive, e.g., drug resistance and immune escape, HIV mutations on viral relative fitness parameters (5, 17, 34, 35, 47). Fundamentally, the assay differs very little from a traditional growth kinetics assay with the exception of the presence of two viruses: viral replication over time is assessed by the repeated sampling of culture supernatant (or cells) and the quantification of virus therein. The advantage of the dual-infection approach is the increased sensitivity to detect small growth differences that results from the direct comparison of viruses that have been cultured, isolated, and quantified under identical conditions. In contrast to, for example, parallel but independent monocultures, the nonspecific variation that is inevitably introduced during manipulation of the assay is controlled because it affects both viruses equally. Although the presence of two viruses in a single culture raises the possibility of competition for target cells, the standard inoculation at a very low MOI makes this unlikely because target cells are present in vast excess of virions, which essentially precludes competition. Similarly, the utility of the assay for quantifying viral fitness parameters derives from the specific quantitative methods used to analyze the growth data, not from any special property of the assay itself. In fact, the very same fitness parameters could be calculated using data generated by traditional, single-virus, growth kinetics assays, albeit with lower resolution because of the "noise" of nonspecific variability as described above.

The major technical hurdle of the dual-infection assay is the requirement for differential quantification of two essentially isogenic viruses from a mixed sample. Our use of synonymous nucleotide substitutions to "tag" the viruses, an approach similar to that recently described by Anastassopoulou et al. (5), provides two advantages over the insertion of heterologous sequence tags (34) or fluorescence genes (17, 50) into the HIV genome. First, the use of synonymous tags avoids the disruption of any viral proteins and allows consideration of the impact of mutations in the context of a native virus. Second, although we placed the synonymous tags in *nef* to be consistent with previous methods, the flexibility to place synonymous tags in close proximity to any mutation of interest can reduce, or in theory eliminate, the potential for recombination between the mutation and the detector tag. Although the use of low-MOI inocula and short-duration infections also results in negligible recombination, the placement of the synonymous tag in close proximity to the mutation of interest would provide for greater experimental flexibility, including assays using high-MOI infection. It is important to note, however, that although it is undesirable, recombination is not a fatal flaw; the rearrangement of mutation-tag linkage does not cause erroneous detection of mutation-specific differences where there are none, i.e., false positives, but rather it decreases the power to detect mutation-specific differences that truly exist, i.e., increases false negatives.

The interpretation of results of in vitro assays of relative fitness parameters suffers from the need to extrapolate the in vitro-derived values to the viral infection in vivo. Although extrapolation is an inherent weakness of any in vitro assay, the quantification of the RRC associated with the antiretroviral drug resistance mutation RT M184V provides a clinically relevant point of reference, or benchmark, against which the values for other mutations can be compared. The RT M184V mutation is well suited for this task because not only has it been demonstrated by in vitro analysis to cause a significant reduction in viral replication capacity (34), but it is also known to contribute to decreased viral loads in vivo. In fact, the impact is so great that an M184V-selective antiretroviral is often continued following the emergence of resistance in order to maintain the presence of the M184V variant in the patient (15, 40, 49). The value of this mutation as a benchmark of replication capacity is further increased by the apparent constancy of its impact; assayed here in the context of a HIV-1C genetic background, the impact on viral replication capacity (RRC = 0.86) is quite comparable to that observed in previous studies in which it was expressed in a HIV-1B genetic background. For example, in the most directly comparable study, Lu and Kuritzkes describe a 7% reduction in replication capacity, approximating an RRC of 0.93, caused by expression of RT M184V in a HIV-1B background (34).

The capacity of HIV to escape from CTL-mediated immune pressure is an important aspect of the pathogenesis of HIV infection and is also likely to challenge any vaccine design

based on eliciting CTL immune responses. The ability to sensitively quantify the impact of CTL escape mutations on HIV replication capacity in a relevant and reproducible manner is an important step in acquiring the data necessary for teasing apart the contributions of multiple, and at times opposing, components of net relative fitness, a driving force of CTL escape dynamics. In this study, we focused our efforts on quantifying the specific impact of several important HLA-B*57/B*5801 escape mutations, individually and in combination, by engineering them into a recombinant virus with a defined genetic background. This approach provides for a very "clean" determination of the intrinsic impact of specific mutations of interest, and as additional CTL-associated mutations in HIV are identified by careful analysis of clinical viral sequences, it will continue to be useful for fine mapping of the relative impacts of such mutations on viral replicative capacity. In addition, however, future studies expanding the approach to include larger segments of clinically derived sequence, e.g., *gag* in toto, will provide important comparators for evaluating the recombinant-derived RRCs. The continued investigation of the mechanisms and dynamics of CTL not only increases our understanding of the factors underlying the clinical progression of HIV infection but also contributes important information toward the eventual development of a durable vaccine construct.

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