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Use of Molecular Beacons for Rapid, Real-Time, Quantitative Monitoring of Cytotoxic T-Lymphocyte Epitope Mutations in Simian Immunodeficiency Virus

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Immune pressure on lentiviruses exerted by cytotoxic T lymphocytes (CTL) selects for virus CTL epitope mutations. Currently employed methods for monitoring emerging CTL epitope mutations rely on the labor-intensive and time-consuming techniques of virus population or clonal sequencing. Here we describe the development of a high-throughput quantitative reverse transcription-PCR assay that facilitates large-scale CTL epitope monitoring. This approach utilizes both sequence-specific molecular beacons and the sequence-independent double-stranded DNA binding dye Sybr Green. We show that this assay detects single-nucleotide mutations in an immunodominant CTL epitope in viral RNA isolated from both viral culture supernatants and plasma samples from simian immunodeficiency virus (SIV)-infected rhesus monkeys. Furthermore, mutant viruses can be detected even when they represent as few as 500 mutant copies in a sample containing 10,000 total copies. This real-time PCR technique for evaluating CTL epitope mutations may prove to be a useful tool for monitoring the genetic drift of human immunodeficiency virus and SIV in infected individuals.

Cytotoxic T lymphocytes (CTL) play an important role in controlling human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication in infected individuals (12, 15, 21). In so doing, they exert significant immune pressure on replicating virus and select for CTL epitope mutations (reviewed in reference 17). The accumulation of such mutations in CTL epitopes can lead to increased virus replication and disease progression. In view of the importance of these events in the clinical progression of AIDS, it will be important to be able to monitor the evolution of CTL epitope mutations as they accumulate in HIV and SIV. Currently employed methods for analyzing CTL epitope nucleotide variation rely either on virus population (1, 3, 16) or clonal sequencing (2, 4, 5, 8, 18, 23). Because these methods are time-consuming and labor-intensive, only a limited number of samples are typically analyzed in studies.

Molecular beacons are stem-loop oligonucleotide probes that contain a target-specific loop that is flanked by self-complementary stems covalently linked to a fluorophore and a quencher at the 5′ and 3′ ends (22). In the absence of a complementary target, the molecular beacon forms a stem-loop structure that brings the fluorophore into close proximity with the quencher, causing fluorescence to be quenched by fluorescence resonant energy transfer. In the presence of a complementary target, the molecular beacon hybridizes to the target, resulting in fluorescence as the fluorophore and quencher molecules are separated from one another. The competition between closed and open forms of the molecular beacon allows the discrimination of nucleotide mutations within the target sequence (13, 14).

Here we describe the development of a quantitative real-time reverse transcription-PCR (qRT-PCR) assay that detects single-nucleotide mutations at all positions of the immunodominant Mamu-A*01-restricted SIV Gag p11C, C-M CTL epitope. This assay combines the sequence-independent double-stranded DNA (dsDNA) dye Sybr Green and sequence-specific molecular beacons spanning the p11C, C-M CTL epitope and quantifies the amount of virus encoding a wild-type CTL epitope as a percent of total virus in a single sample. We show that this assay detects single-nucleotide mutations in the p11C, C-M CTL epitope in viral RNA isolated both from culture supernatants and plasma samples from SIV-infected rhesus monkeys.

MATERIALS AND METHODS

Plasmids and viruses. Mutant *gag* plasmids were created by PCR mutagenesis using the QuickChange kit (Stratagene, La Jolla, California) with the previously described p239SpSp5′ and SIVsmE543 plasmids (a generous gift from Vanessa Hirsch) (9) as the template. p239SpSp5′ was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, from Ronald Desrosiers (10, 20). The oligonucleotide primers T47IF (5′-CAGGCACTGTCAGAAGGTTGCATCCCCTATGACATTAATCAGATGTTAAATTG-3′, nucleotides 1831 to 1883; here and below, underlined letters represent the codon that was mutated) and T47IR (5′-CAATTTAACATCTGATTAATGTCATAGGGGATGCAACCTTCTGACAGTGCCTG-3′, nucleotides 1831 to 1883) were used to change the SIVmac239 Thr (ACC) to Ile (ATC). The oligonucleotide primers T47SF (5′-CAGGCACTGTCAGAAGGTTGCTCCCCCTATGACATTAATCAGATGTTAAATTG-3′) and T47SR (5′-CAATTTAACATCTGATTAATGTCATAGGGGGAGCAACCTTCTGACAGTGCCTG-3′) were used to change the SIVsmE543 Thr (ACC) to Ser (TCC).

Plasmids for standard curves were generated by PCR amplifying a fragment of the *gag* gene from wild-type and mutant plasmids encoding the SIVmac239 and SIVsmE543 provirus. The SIVmac239 *gag* plasmids were PCR amplified using SIVmac239 forward and SIVmac239 reverse primers (Table 1) and wild-type and mutant p239SpSp5′ as templates. The SIVsmE660 *gag* plasmids were PCR am-

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TABLE 1. SIV *gag*-specific primers and molecular beacons used in the SIV qRT-PCR assay[a]

Primer or beacon	Sequence	Fragment (bp)[d]
SIV mac239 forward	ATACTGTCTGCGTCATCTG	
SIV mac239 reverse	CCTAAGTTGTCCTTGTTGTG	449
SIV mac239 beacon	G[b]CGCATGTGCACCCCCTATGACATTAATCAGATGCATGCGC[c]	
SIV smE660 forward	AGACATCTAGTGGTGGAAACAG	
SIV smE660 reverse	CTAAGTTGCCCTGCTGGTAG	374
SIV smE660 beacon	G[b]CGCATGACTCCCTATGACATCAATCAAATGCTACATGCGC[c]	

[a] Sequences of primers and molecular beacons are shown in 5′ to 3′ orientation. Beacons were designed to hybridize to the antisense strand. Underlined sequences represent the complementary sequence required for hairpin formation.
[b] Nucleotide to which black hole quencher 3 (BHQ-3) is coupled.
[c] Nucleotide to which Quasar 670 is coupled.
[d] Expected size of amplicon using sequence-specific forward and reverse primers.

plified using SIVsmE660 forward and SIVsmE660 reverse primers (Table 1) and wild-type and mutant SIVsmE543 provirus plasmids as templates. Plasmids were subjected to PCR following the manufacturer's protocol using 50 ng of plasmid, 50 pmol of primers, 2 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and 2.5 U of Ampli*Taq* Gold (Applied Biosystems, Foster City, California) in a 50-μl reaction volume. The cycling parameters were 95°C for 10 min and 40 cycles of 95°C for 60 s, 55°C for 30 s, and 72°C for 1 min. Amplicons were gel purified from a 2% agarose gel using the QIAquick gel extraction kit (QIAGEN, Valencia, California) and TA cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, California).

Quantitative PCR and RT-PCR assay. Wild-type and mutant plasmids were purified using the QIAGEN plasmid maxi kit (Valencia, California) and diluted to a concentration of 10^7 to 10^1 plasmid copies/μl in water containing 1 μg/ml *Escherichia coli* DNA as the carrier. Standard curves were established by analyzing serial dilutions of wild-type and mutant plasmids from 10^8 to 10^2 copies per tube. Quantitative PCR was performed by subjecting plasmid standards in duplicate to PCR with the Brilliant Sybr Green kit (Strategene, La Jolla, California) following the manufacturer's protocol using 200 nmol\ of primers and 100 nmol of molecular beacon (Table 1) in a 25-μl reaction volume. Oligonucleotide primers were manufactured by Biosource (Camarillo, California), and molecular beacons were designed using Beacon designer 2.0 (Premier BioSoft, Palo Alto, California) and manufactured with a 7-bp stem by Biosearch Technologies (Novato, California). The cycling parameters employed in this amplification were 95°C for 10 min and 40 cycles of 95°C for 60 s, 55°C for 30 s, 57°C for 30 s, and 72°C for 1 min.

For quantitative RT-PCR, SIV RNA was isolated from 500 μl of plasma or 140 μl of culture supernatant using a QIAmp Viral RNA mini kit (QIAGEN, Valencia, California). cDNA was synthesized in duplicate from 10 μl of viral RNA

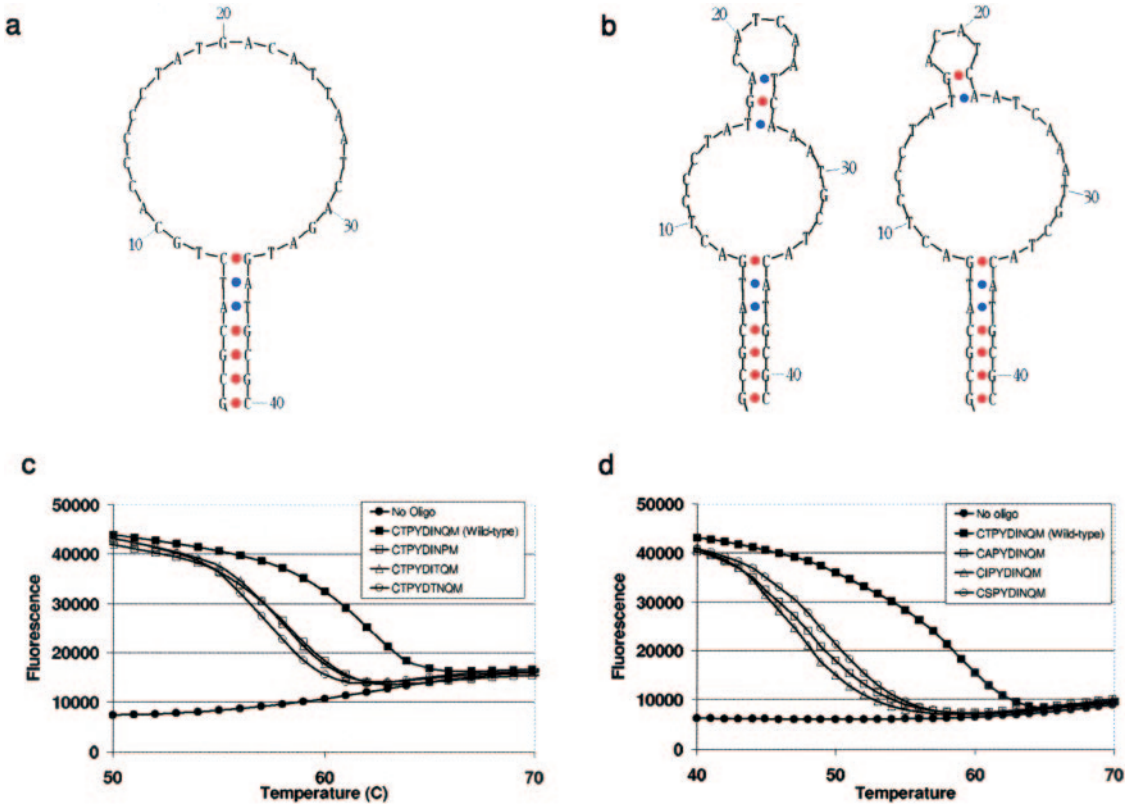

FIG. 1. SIV Gag p11C, C-M CTL epitope beacons can detect single-nucleotide mutations. (A and B) The most stable predicted secondary structures of the SIVmac239 and SIVsmE660 p11C, C-M molecular beacons, respectively. Secondary structures were determined using the mfold server (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). (C and D) The specificities of the SIVmac239 and SIVsmE660 p11C, C-M molecular beacons were tested against oligonucleotides of wild-type sequences (■) or oligonucleotides with a single-nucleotide mutation at the indicated position. Oligo, oligonucleotide.

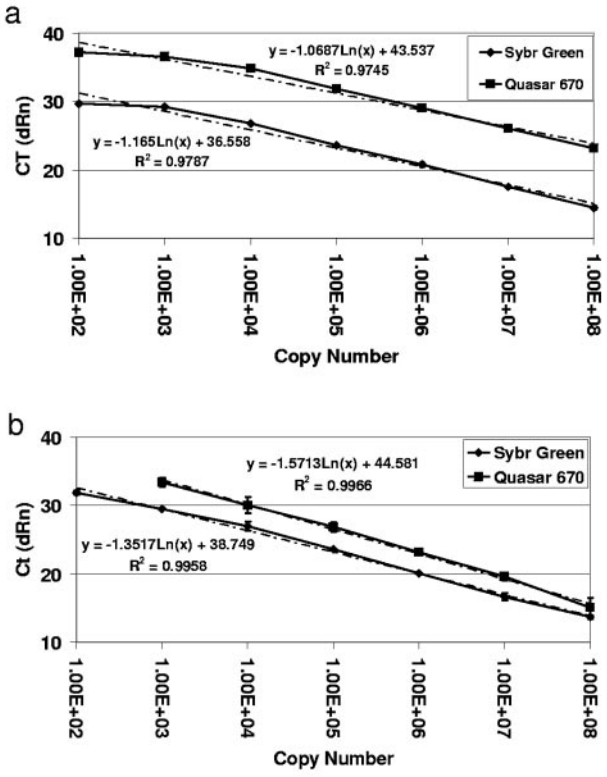

FIG. 2. Linearity of a dilution of plasmid standards over 7 logs. Serial dilutions from 1×10^8 to 1×10^2 of wild-type plasmid standards were tested in triplicate for SIVmac239 p11C, C-M beacon (A) and SIVsmE660 p11C, C-M beacon (B). dRn, baseline corrected normalized fluorescence.

(50°C for 30 min) and then subjected to PCR in a single tube following the manufacturer's protocol in the Brilliant Sybr Green QRT-PCR kit (Strategene, La Jolla, California), using the same cycling parameters as described above for the quantitative PCR assay.

To confirm that the Sybr Green fluorescence for each sample was the result of specific target sequence amplification, thermal denaturation curves were generated for all samples following the 40 cycles of PCR. The dissociation curve cycling parameters employed were 30 s at 55°C followed by successive 1°C increases for 41 cycles to a final temperature of 96°C. Fluorescence was measured three times for the last 21 s of each cycle.

Reactions were performed using a Stratagene Mx4000 Multiplex Quantitative PCR system, and fluorescence data were collected continuously during the 57°C step. All data analysis was performed with the Mx4000 v3.00 software (Stratagene, La Jolla, California) using the moving average and adaptive baseline algorithm enhancements. Fluorescence was measured in each well during every cycle of PCR, and the background threshold fluorescence for the Sybr Green signal was calculated by the Mx4000 instrument. The mutant p11C, C-M *gag* standard curves were analyzed to determine the background threshold fluorescence value. This value was manually set so that the Quasar 670 fluorescence of the mutant standards was below the threshold fluorescence value. This value was in the range of 0.05 and 0.1 fluorescence units. These threshold fluorescence values were then used to analyze the wild-type and experimental samples, and initial template quantities were calculated using the Mx4000 software algorithms. An increase in fluorescence over the calculated background fluorescence indicates amplification of the target sequence. If no increase in fluorescence is observed after 40 cycles of amplification, the sample is assumed to be negative. The percent wild-type p11C, C-M copy number was calculated as follows: total virus copy number (Sybr Green signal)/wild-type copy number (Quasar 670 signal) × 100.

Viral replication kinetics. Recombinant SIVmac239 viruses were produced as previously described (10, 20). Briefly, 5 μg of each proviral half was digested with SphI, phenol-chloroform extracted, ethanol precipitated, and ligated. The ligation mix was then transfected into 2×10^6 CEMx174 cells (American Type Culture Collection) by the DEAE-dextran method for 1 h. Recombinant SIVsmE543 viruses were produced by transfecting 10 μg of proviral SIVsmE543 plasmid DNA into 2×10^6 CEMx174 cells by the DEAE-dextran method for 1 h. Cells were then washed three times with phosphate-buffered saline and incubated at 37°C for 1 h in RPMI 1640 complete medium plus 10% fetal calf serum, penicillin, streptomycin, and 100 U of DNase to remove residual plasmid DNA. Cells were then washed twice and incubated at 37°C in RPMI 1640 complete medium plus 10% fetal calf serum, penicillin, and streptomycin. One milliliter of sample was collected every day for 10 days. Samples were centrifuged for 5 min at 5,000 × *g* to remove cells and debris, and 0.75 ml of sample was removed and stored at −20°C until analyzed. Cultures were monitored for p27 expression by enzyme-linked immunosorbent assay (Coulter, Miami, Florida) and for p11C, C-M epitope reversion by the qRT-PCR beacon assay.

Virus sequencing. Five units of Ampli*Taq* gold was added to qRT-PCRs and incubated at 95°C for 10 min and 72°C for 10 min to ensure that PCR products have 3′-A overhangs. Reactions were then gel purified from a 2% agarose gel (QIAGEN, Valencia, California) and TA cloned (Invitrogen, Carlsbad, California) into the pCR 2.1 vector. Individual transformed colonies were subjected to T7 dideoxy sequencing.

RESULTS

Because currently available methods for monitoring viral epitope variation are too labor-intensive to be broadly applicable, we sought to develop a rapid assay for quantifying virus encoding wild-type and variant epitope sequences in single samples. To do so, we combined sequence-specific molecular beacons and the sequence-independent dsDNA binding dye Sybr Green with viral RNA and performed single-tube quantitative RT-PCR. In theory, the Sybr Green should bind to all amplicons regardless of sequence, while the molecular beacon should bind only to amplicons with a wild-type p11C, C-M epitope sequence. The exquisite sensitivity of molecular beacons relies on a competition between the closed and open forms of the molecule (22). In the absence of complementary base pairing, the closed form is favored, driving the formation of a stem-loop structure and bringing the fluorophore (Quasar 670) into close proximity with the quenching dye (black hole quencher 3). In the presence of complementary base pairing, the molecular beacon assumes an open conformation that separates the fluorophore from the quencher dye, resulting in fluorescence.

We developed this qRT-PCR assay for use with two related and commonly studied SIVs, SIVmac239 and SIVsmE660. Because the *gag* genes of SIVmac239 and SIVsmE660 are only 87% identical in nucleotide sequence and 89% identical in amino acid sequence (9) and because the Gag p11C, C-M CTL epitopes (CTPYDINQM) of these two viruses differ by three nucleotides, two different molecular beacons were designed. The molecular beacons were designed to hybridize to the entire 27 nucleotides of the Gag p11C, C-M CTL epitope so they might detect sequence changes at all positions of the epitope (Table 1). However, because the Beacon Designer software predicted that the SIVsmE660-specific molecular beacon spanning this sequence was suboptimal, this molecular beacon was shifted by three nucleotides in the 3′ direction. Thus, the SIVmac239 molecular beacon hybridized to the nucleotide sequence encoding the entire p11C, C-M CTL epitope (CTPY DINQM), while the SIVsmE660 molecular beacon hybridized to the nucleotide sequence encoding eight amino acids of the epitope plus one additional amino acid (TPYDINQML).

We next characterized these two SIV Gag p11C, C-M-spe-

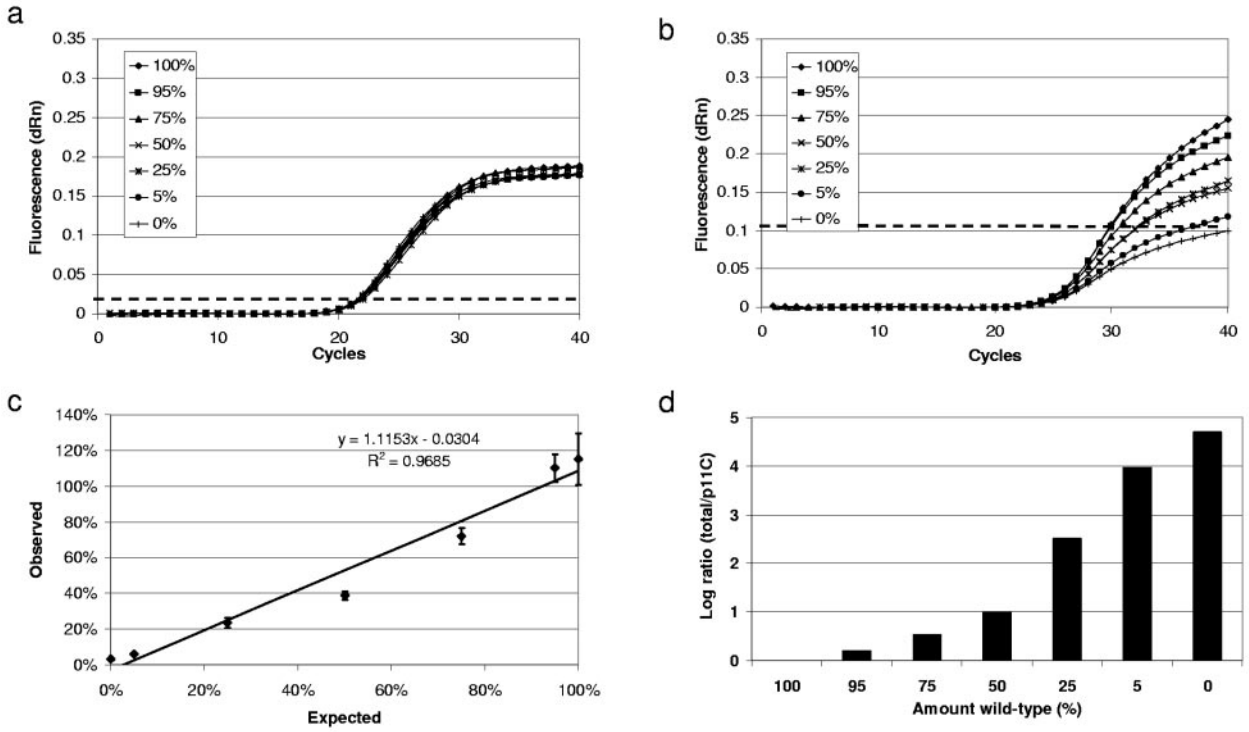

FIG. 3. Quantitative PCR curves to measure SIVmac239 p11C, C-M beacon sensitivity. Sensitivity was measured using 10^4 total copies of SIVmac239 plasmids spiked with different percentages of mutant SIVmac239. Sybr Green (A) and Quasar 670 (B) fluorescences were measured for all samples during the annealing step of each reaction. The threshold value is indicated by a dashed line. (C) Samples spiked with different percentages of wild-type and mutant SIVmac239 plasmids at a total copy number of 10^4 (expected) were analyzed using the Stratagene Mx4000 software package (observed). (D) Total and wild-type copy numbers were determined using the Stratagene Mx4000 software package and converted to a log ratio for each sample (log total copies − log wild-type copies).

cific molecular beacons. The predicted secondary structures of the two p11C, C-M-specific molecular beacons suggested that they both would assume a closed conformation in the absence of complementary oligonucleotides (Fig. 1A and B). To determine the optimal annealing temperature for each molecular beacon, a melting curve analysis was performed. This analysis was performed using oligonucleotides complementary to the molecular beacon or oligonucleotides with single nucleotide substitutions at several different positions (Fig. 1C and D). The optimal annealing temperature for both the SIVmac239- and SIVsmE660-specific p11C, C-M molecular beacon was found to be 57°C. This temperature was determined by the Stratagene Mx4000 software to produce the highest signal-to-noise ratio and was used for all subsequent experiments.

To evaluate the sensitivity of the assay, serial dilutions from 1×10^8 to 1×10^2 copies of wild-type SIVmac239 (Fig. 2A) and SIVsmE660 (Fig. 2B) *gag* plasmids were analyzed. We observed a linear relationship with Sybr Green between the copy number and cycle threshold (Ct) value through 7 logs of sample dilutions for both SIVmac239 and SIVsmE660. Seven logs of sensitivity were observed with the SIVmac239 molecular beacon, and 6 logs of sensitivity were observed with the SIVsmE660 molecular beacon.

The specificity of the beacons was analyzed using samples with a fixed amount of total *gag* plasmid but variable relative amounts of wild-type and mutant *gag* plasmids. Samples contained 100%, 95%, 75%, 50%, 25%, 5%, or 0% wild-type SIVmac239 *gag* plasmid, each sample supplemented with p11C, C-M T47I mutant SIVmac239 *gag* plasmid to reach a total amount of 1×10^4 copies of *gag* plasmid. Regardless of how much wild-type plasmid was in a sample, an increase in Sybr Green fluorescence was detected in all samples at cycle 20, and this fluorescence increased comparably in intensity with each successive cycle of PCR (Fig. 3A).

While molecular beacons provide a high level of sequence specificity, their ability to discriminate between single-nucleotide mutations is highly temperature dependent. Although an optimal annealing temperature of 57°C was obtained for each molecular beacon, this annealing temperature yielded some background fluorescence in the presence of an oligonucleotide containing any number of single-nucleotide mutations (Fig. 1C and D). To take into account the low level of background fluorescence, the Quasar 670 fluorescence threshold had to be set to a level higher than was used for Sybr Green. This arbitrary threshold level was determined by using a sample with 1×10^4 copies of T47I mutant SIVmac239 *gag* plasmid. When the appropriate background fluorescence threshold was set for the Quasar 670 fluorescence, the molecular beacon signal for each sample was found to be directly proportional to the amount of wild-type *gag* plasmid present in each sample (Fig. 3B and C). The combination of both Sybr Green and the molecular beacons allowed for the precise quantification of the amount of wild-type virus as a percent of the total virus present in a sample. Furthermore, the Sybr Green signal served as an

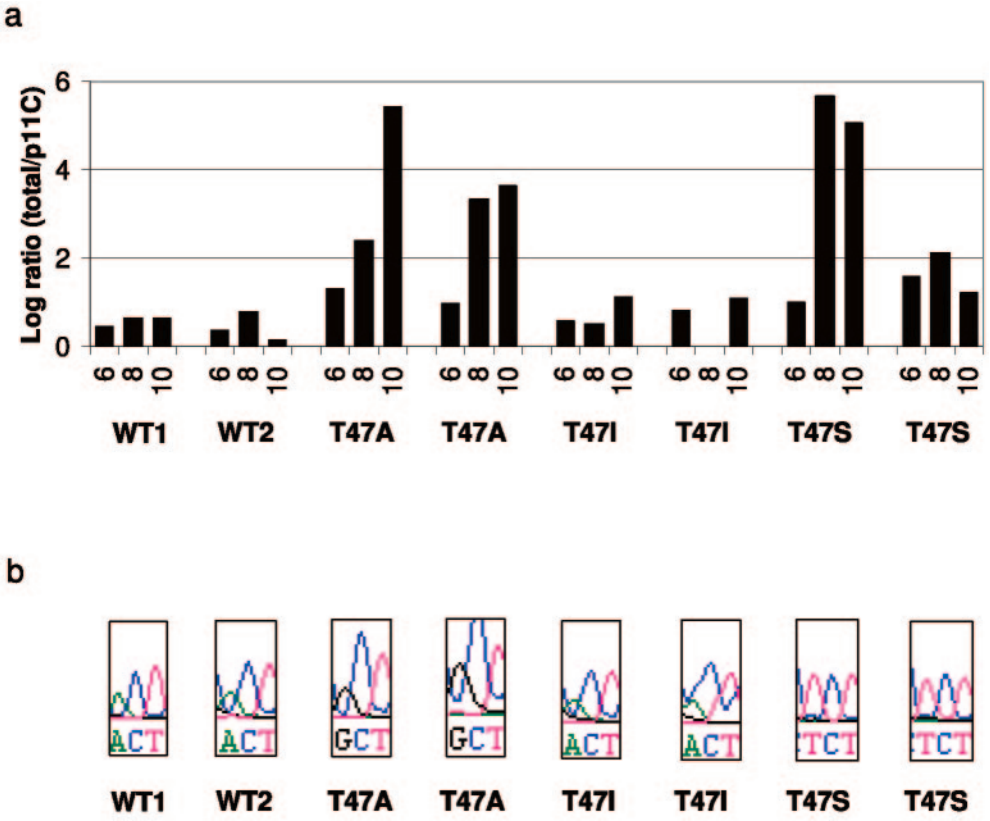

FIG. 4. Analysis of the p11C, C-M CTL epitopes from wild-type and mutant SIVmac239 viruses during in vitro infection of CEMx174 cells. CEMx174 cells were transfected with wild-type and mutant SIVmac239 proviral plasmids. Viral RNA was isolated and analyzed from supernatants on days 6, 8, and 10 following transfection. (A) The log ratio was calculated for each sample. (B) Day 8 PCRs were purified, TA cloned, and sequenced. WT, wild type.

internal control and allowed for the generation of dissociation curves for all samples. The dissociation curves were identical for all samples (data not shown), indicating that amplification of the target sequence occurred regardless of the wild-type-to-mutant-plasmid ratio.

While the use of percentages is a practical method for quantifying the amounts of wild-type and mutant viruses in a mixed virus population, it is not ideal. Subtle variations at the lower limits of detection may be exaggerated due to the differences in sensitivity between Sybr Green and the molecular beacons. Therefore, we calculated the log ratio for each of the mixed plasmid samples (Fig. 3D). The log ratio was calculated as $\log_{10}$(total copy number) − $\log_{10}$(wild-type copy number). The use of the log ratio did not affect the ability to detect the mutant plasmids, even when the mutant plasmids were present at low levels.

Because mutations frequently arise in virus propagated in vitro, we sought to use the assay to track the reversion of previously described p11C, C-M CTL epitope mutations that have been shown to alter the fitness of replicating viruses (6, 19). We transfected CEMx174 cells with either wild-type or mutant (T47A, T47I, and T47S) SIVsmE543 proviral plasmid DNA and monitored the p11C, C-M epitope sequence of emerging virus on days 6, 8, and 10. By day 6 posttransfection, all cultures had virus titers of greater than 1×10^4 copies/ml (data not shown). Cells transfected with the wild-type proviral plasmid DNA had a low-to-undetectable log ratio at all time points, which suggested that little or no sequence variation was present. Cells transfected with the T47A and T47S mutant proviral plasmids had low log ratios on day 6 that increased on days 8 and 10, while cells transfected with the T47I mutant proviral plasmid had low log ratios at all time points (Fig. 4A). These data suggested that the CTL epitope in the T47A and T47S viruses remained mutant while a reversion event occurred in the T47I virus. To confirm these findings, viral RNA was isolated on day 8 from each culture, TA cloned, and analyzed by direct sequencing. As was suggested by the beacon assay, virus isolated from cells transfected with the T47A and T47S proviral plasmids encoded the original mutations while virus from cells transfected with the wild-type and T47I proviral plasmids encoded a threonine (ACT) at position 47.

We next sought to validate the assay using virus isolated from plasma samples of SIVsmE660-infected rhesus monkeys in which the virus quasispecies sequence had been previously determined by direct sequencing (4). All viral RNA sequences of the p11C, C-M epitope for monkey J8N were wild type at week 11 following infection (pre-escape) and contained a single-nucleotide mutation at week 43 following infection (post-escape). Plasma viral RNA was also analyzed from monkey R468 at week 11 following infection (mix-escape) in a sample in which it had previously been determined there was a mixture of wild-type (15 of 16 sequences) and mutant (1 of 16 se-

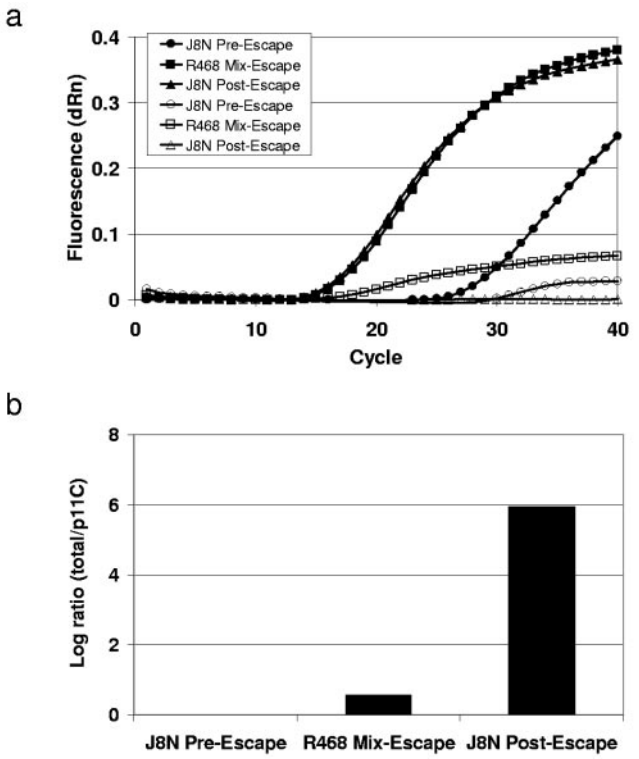

FIG. 5. qRT-PCR curves from pre- and post-escape plasma viral RNA samples for SIVsmE660-infected rhesus monkeys. Viral RNA was isolated from plasma of SIVsmE660-infected rhesus monkeys and analyzed using the SIVsmE660 p11C, C-M beacon assay. Closed symbols, Sybr Green fluorescence; open symbols, Quasar 670 fluorescence. dRn, baseline corrected normalized fluorescence.

quences) p11C, C-M epitope sequences. When samples were analyzed using the beacon assay, a Sybr Green signal was detected for all samples, indicating that SIV RNA was present, albeit in different quantities (Fig. 5). Molecular beacon signals were observed from both the J8N pre-escape and the R468 mix-escape samples, while no signal was detected from the J8N post-escape sample (Fig. 5). Quantification of the signals revealed an absolute concordance between the results of the beacon assay and direct viral sequencing for monkey J8N (Table 2). A close concordance between the results of the beacon assay and direct viral sequencing was observed for samples from monkey R468 (Table 2). Results from the beacon assay indicated that 27% of the virus in the sample was wild type, while direct sequencing revealed that 93% of the virus in the sample (15 of 16 clones) was wild type. This discrepancy observed between the results of the beacon assay and direct viral sequencing may have arisen because of the limited number of clones that were sequenced. Nonetheless, the qRT-PCR beacon assay was capable of detecting the presence of mutant p11C, C-M CTL epitope sequences in plasma viral RNA samples.

DISCUSSION

Currently employed methods for monitoring genetic variation in viral CTL epitopes require the labor-intensive sequencing of multiple clones. Here we describe the development of a high-throughput quantitative RT-PCR assay for analyzing nucleotide diversity in the immunodominant Gag p11C, C-M CTL epitope in two strains. The combination of both molecular beacons and Sybr Green in a single reaction enables the quantification of virus encoding wild-type p11C, C-M CTL epitope nucleotide sequence as a percent of the total amount of virus present in a sample. The results demonstrate that p11C, C-M CTL epitope variation can be detected in virus isolated from plasma samples of SIV-infected rhesus monkeys in less than 4 h and that these results are concordant with direct viral sequencing. This assay is sensitive and highly specific. Single-nucleotide mutations are reliably detected even when present at low levels relative to the wild type. While the specific sequence of the mutant CTL epitope cannot be determined using this assay, samples that are positive for CTL epitope variation can be rapidly TA cloned into sequencing vectors to determine the precise nucleotide substitutions that have arisen.

While this technique requires the development of epitope-specific molecular beacons and relies on having both wild-type and mutant control plasmids, today's arsenal of molecular biology techniques allows for the development of such reagents in a relatively short period of time. Furthermore, the determination of which nucleotides in a given CTL epitope are prone to mutation has been facilitated by the rapidly growing HIV sequence database and ongoing efforts in the identification of CTL epitopes. Therefore, this sensitive and highly specific assay could easily be adapted to monitor the evolution of any number of different HIV and SIV CTL epitope sequences.

HIV and SIV CTL epitope sequences are in constant flux in an infected individual as a result of selection pressures associated with escape from immune recognition on one hand and requirements for viral fitness on the other hand (7, 11). This

TABLE 2. Analysis of pre- and post-escape plasma viral RNA from SIVsmE660-infected rhesus monkeys

Criterion	Sample	Trial 1	Trial 2	Avg	SD
Total copy number	J8N pre-escape	1.05×10^4	$1.78E \times 10^4$	1.41×10^4	5.18×10^3
	R468 mix-escape	6.52×10^7	7.70×10^7	7.11×10^7	8.34×10^6
	J8N post-escape	9.13×10^7	8.77×10^7	8.95×10^7	2.60×10^6
Wild-type copy number	J8N pre-escape	9.72×10^3	1.87×10^4	1.42×10^4	6.37×10^3
	R468 mix-escape	1.88×10^7	1.98×10^7	1.93×10^7	7.14×10^5
	J8N post-escape	0	0	0	0
% Wild type	J8N pre-escape	92.98	105.34	99	9
	R468 mix-escape	28.85	25.75	27	2
	J8N post-escape	0.00	0.00	0	0

results in the continuous emergence of quasispecies of virus with mutations in epitopes that can escape recognition by CTL. However, because of decreased fitness, these viruses then can revert back to wild-type sequences if immune selection pressure decreases (6, 7). It will be important to gain a better understanding of the strategies used by HIV and SIV to balance mutations associated with viral escape from CTL and the fitness costs associated with those mutations.

While current techniques allow for a detailed analysis of the immune response to multiple CTL epitopes during large-scale clinical trials and monkey studies, traditional methods of virus population and clonal sequencing are limited for closely monitoring the genetic drift of HIV and SIV during these studies. In addition, small sample volumes limit the number of epitopes that can be studied at one time. This high-throughput quantitative RT-PCR assay overcomes the current virus-sequencing bottleneck associated with large-scale CTL epitope monitoring. Furthermore, while the assay is currently limited to analysis of a single CTL epitope, multiplexing with additional molecular beacons will allow for the simultaneous analysis of multiple CTL epitopes in a single reaction. The detailed monitoring of immune responses in conjunction with CTL epitope variation will provide a better understanding of how HIV and SIV respond to changes in immune selection pressures.

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