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The G490E mutation in reverse transcriptase does not impact tRNA primer selection by HIV-1 with altered PBS and A-loop

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Abstract

The initiation of HIV-1 reverse transcription utilizes a cellular $tRNA^{Lys,3}$ as a primer. The 3′ terminal 18-nucleotides of the cellular $tRNA^{Lys,3}$ are complementary to a region on the viral genome, designated as the primer binding site (PBS). Previous studies have shown that forcing HIV-1 to utilize alternative tRNA primers through alteration of the PBS results in viruses that revert to utilize $tRNA^{Lys,3}$ following in vitro replication. In some instances though, HIV-1 has been shown to select alternative tRNAs for initiation of reverse transcription if additional mutations upstream in the U5 region (A-loop) were made to be complementary to these alternative tRNAs. Recently, an HIV-1 has been described in which the U5 region distinct from the A-loop, designated as the primer activation site (PAS), was mutated in conjunction with the PBS to force the virus to use $tRNA^{Lys1,2}$ as a primer. An additional mutation in reverse transcriptase (RT), G490E, was found to facilitate the forced use of $tRNA^{Lys1,2}$ as the primer. In the current study, we have investigated the impact of the G490E mutation in RT on the selection and use of alternative primers by HIV-1. Viruses were first constructed in which the PBS and A-loop region were made complementary to $tRNA^{Trp}$. Previous studies from our laboratory have shown that these viruses are unstable and mutate to select $tRNA^{Met}$ or $tRNA^{Lys1,2}$. Analysis of the replication of viruses with the U5 and PBS complementary to $tRNA^{Trp}$ with or without the G490E mutation revealed no significant differences with respect to infectivity and viral growth in SupT1 or peripheral blood mononuclear cells (PBMC). In addition, the presence of the G490E mutation did not influence the capacity of this virus to revert to utilize $tRNA^{Met}$ as the primer for initiation of reverse transcription. In a previous study, we have described an HIV-1 that has been forced to utilize $tRNA^{Lys1,2}$ through mutations in the A-loop and PBS. The G490E RT mutation in this virus did not impact on the virus infectivity or growth in SupT1 or PBMC. We did not find a significant fitness advantage to viruses in which the A-loop and PBS were made complementary to $tRNA^{Lys1,2}$ that also contained the G490E mutation in RT. The results of these studies then establish that HIV-1 can be forced to use alternative primers through mutations in the U5 (PAS or A-loop) for certain tRNAs. Furthermore, for mutations in the A-loop and PBS, the RT does not play an important role in dictating the selection of the alternative primers to be used for initiation of reverse transcription.

Keywords: tRNA; Reverse transcriptase; HIV-1; Primer binding site

Introduction

Reverse transcription of the retroviral RNA genome into a DNA intermediate is an essential step in virus replication. Initiation of reverse transcription occurs near the 5′ end of the viral genome at a site designated as the primer binding site (PBS) (Panet and Berliner, 1978; Peters and Dahlberg, 1979; Temin, 1981). The PBS is complementary to the 3′ terminal 18-nucleotides of the cellular tRNA that is used as the primer. The tRNA that is selected from the intracellular milieu for use as a reverse transcription primer is highly conserved amongst different retroviruses (Mak et al., 1997; Marquet et al., 1995). For example, human immunodeficiency virus (HIV-1) exclusively uses $tRNA^{Lys,3}$ for initiation of reverse transcription. The mechanism of how retroviruses select particular tRNAs probably involves multiple mechanisms. Previous studies with HIV-1 have found that the Gag and Gag-Pol proteins are assembled into virus-like particles which are enriched for $tRNA^{Lys1,2}$ and $tRNA^{Lys,3}$ (Kohorchid et al., 2000; Mak et al., 1994). Recent studies have shown that lysl tRNA synthetase is

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also enriched in virions, suggesting that this protein could chaperone $tRNA^{Lys1,2,3}$ into virions (Cen et al., 2002; Javanbakht et al., 2003; Jiang et al., 1994).

Although $tRNA^{Lys1,2,3}$ are enriched in HIV virions, previous studies have shown that HIV-1 has the capacity to select and use alternative (non-$tRNA^{Lys,3}$) primers for replication (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995). In early studies, it was found that alteration of the PBS to be complementary to a variety of primers resulted in the virus able to select and use these primers for a limited time of in vitro culture. Eventually, all of these viruses reverted back to utilize $tRNA^{Lys,3}$. Follow-up studies found that additional mutations upstream of the PBS in a region designated as the A-loop allowed the virus to stably utilize some but not all alternative tRNAs that were examined (Kang and Morrow, 1999; Kang et al., 1997, 1999; Wakefield et al., 1996; Zhang et al., 1998). As a consequence of forcing HIV-1 to utilize these alternative tRNAs, the resultant viruses all exhibited a reduced growth as compared to the wild-type virus. Thus, even under conditions designed to promote the use of alternative tRNAs, including $tRNA^{Lys1,2}$ which is enriched in virions, HIV-1 has evolved towards a preference for the selection and use of $tRNA^{Lys,3}$ as the primer for high-level replication.

In a previous study, we reported on the construction and characterization of an HIV-1 which stably utilizes $tRNA^{Lys1,2}$ rather than $tRNA^{Lys,3}$ as the primer for reverse transcription (Kang and Morrow, 1999; Kang et al., 1999). This virus was initially derived from a culture of HIV-1 in which the PBS and A-loop region had been mutated to be complementary to $tRNA^{Met}$ (Kang et al., 1997). Viruses which use $tRNA^{Met}$ were identified from cultures in which the PBS and A-loop had been mutated to correspond to $tRNA^{Trp}$ (Kang et al., 1996). Thus, the parent virus for subsequent viruses that use $tRNA^{Lys1,2}$ or $tRNA^{Met}$ was derived from the in vitro culture of viruses in which the PBS and A-loop were mutated to be complementary to $tRNA^{Trp}$ (Kang and Morrow, 1999; Kang et al., 1996, 1997, 1999). The results from these studies highlight some of the complex evolution that can occur following modification of the PBS. In a recent study, a second HIV-1 virus that utilizes $tRNA^{Lys1,2}$ for initiation of reverse transcription was described (Abbink et al., 2004). For this study, the authors again made the PBS complementary to $tRNA^{Lys1,2}$ but mutated a region upstream of the A-loop designated as the primer activation signal (PAS) to be complementary to $tRNA^{Lys1,2}$. The PAS is complementary to the T φ C arm of $tRNA^{Lys,3}$. The PAS does not contribute to tRNA-PBS annealing but is essential for initiation of tRNA primed reverse transcription. In vitro studies have demonstrated that HIV-1 primer usage can be switched from $tRNA^{Lys,3}$ to $tRNA^{Lys1,2}$ by the use of RNA templates containing the PAS and PBS double mutations (Beerens and Berkhout, 2002). Analysis of HIV-1 with PAS and PBS complementary to $tRNA^{Lys1,2}$ revealed that following extensive long-term in vitro culture HIV-1 could stably utilizes $tRNA^{Lys1,2}$ for replication. An additional mutation in the virus was found within the RT gene of HIV, a G490E mutation in the RT (RNase H) gene facilitated the virus replication and use of $tRNA^{Lys1,2}$ as the primer for replication.

In a current study, we wanted to determine whether the G490E mutation in RT would effect the selection of tRNA primers in HIV-1 in which the A-loop and PBS had been mutated to correspond to alternative tRNAs. Our first study utilized an HIV-1 in which the PBS and A-loop were mutated to be complementary to $tRNA^{Trp}$, since our previous studies have suggested that this virus would naturally evolve to utilize $tRNA^{Met}$ and possibly $tRNA^{Lys1,2}$ following in vitro culture (Kang et al., 1996, 1997). We also determined the effect of the G490E mutation in RT on the replication of HIV-1 in which we have engineered to utilize $tRNA^{Lys1,2}$ through simultaneous mutations in the A-loop and PBS (Kang et al., 1999). The results of our studies demonstrate that for viruses which contain A-loop and PBS mutations, the G490E mutation in RT does not substantially impact on selection of primer tRNA and virus replication. The results are discussed with respect to the importance of the U5 region in the selection of the tRNA primer used for HIV-1 replication.

Results

Effect of G490E mutation in RT on capacity of HIV-1 to select an alternative primer for replication

In a previous study, we described construction and characterization of HIV-1 proviral genomes in which the PBS and A-loop region were mutated to be complementary to $tRNA^{Trp}$ (Kang et al., 1996). These proviral constructs used the HXB2 backbone, and the virus replication was analyzed in SupT1 cells. We found that viruses in which only the PBS was mutated to be complementary to the 3′ terminal nucleotides of $tRNA^{Trp}$ reverted to utilize $tRNA^{Lys,3}$ following short-term in vitro replication in SupT1 cells. In contrast, viruses which contained both the A-loop and PBS complementary to $tRNA^{Trp}$ were unstable but reverted to utilize $tRNA^{Met}$ following in vitro culture. Subsequent characterization of the virus which uses $tRNA^{Met}$ revealed the potential to revert to use $tRNA^{Lys1,2}$ then back to $tRNA^{Lys,3}$ (Kang et al., 1997). In a recent study, we have further characterized the replication of the virus which utilizes $tRNA^{Met}$ (Moore-Rigdon et al., 2005). In this case, we found that viruses derived from NL-4 in which the PBS alone was altered to be complementary to $tRNA^{Met}$ were stable upon replication in either SupT1 or PBMC. Given the interesting and diverse phenotypes of the viruses which use $tRNA^{Trp}$, we wanted to ascertain the effects of the G490E mutation in RT for effects that it would have on the growth of these viruses and potential to revert and utilize an alternative tRNA. Viruses were constructed using the NL-4 provirus in which the PBS and A-loop were mutated to be complementary to the 3′ terminal nucleotides and anticodon region respectively of $tRNA^{Trp}$. We also engineered the G490E mutation into the RT gene of these viruses and a wild-type control virus (Fig. 1A).

We first analyzed the infectivity of these viruses following transfection into 293 T cells. Virus supernatant was assayed for the capacity to infect the JC53BL indicator cell line and infectivity determined as previously described (Moore-Rigdon et al., 2005). The infectivity obtained from transfection of wild-

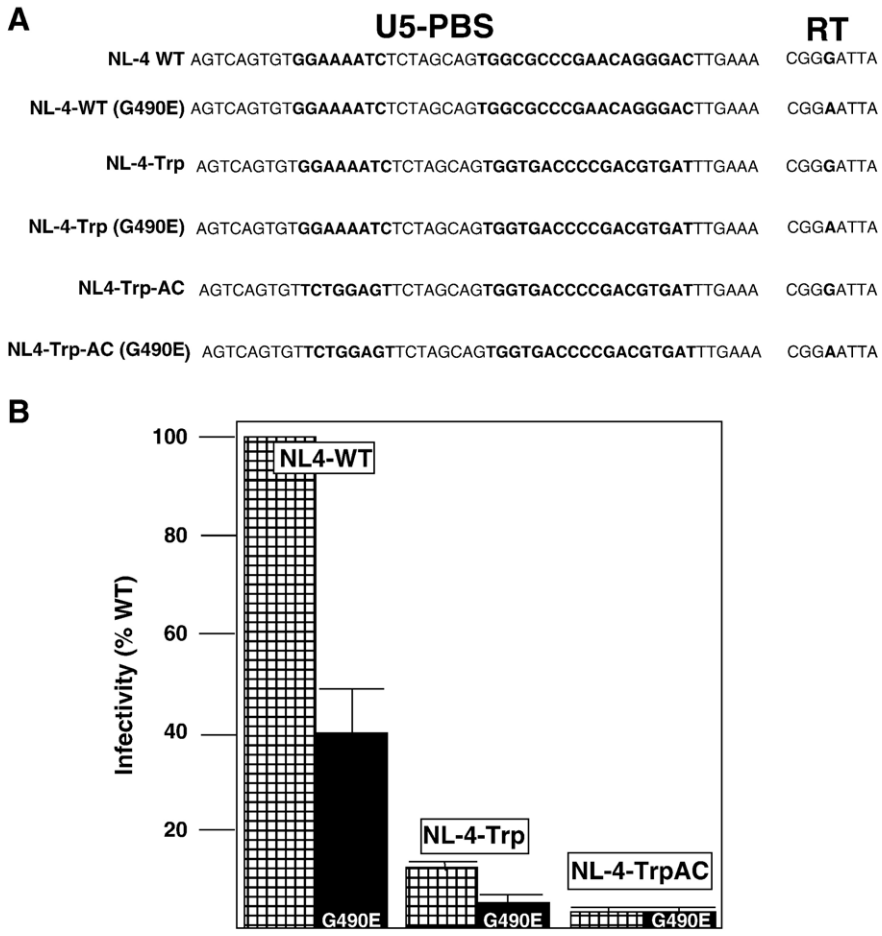

Fig. 1. Characterization of HIV-1 with PBS complementary to $tRNA^{Trp}$. (A) Sequences of HIV-1 and U5-PBS complementary to $tRNA^{Trp}$ and G490E mutation in RT. The proviral backbone NL-4 (NL-4-WT) was used for the construction genomes. The sequences in bold (GGAAAAT) correspond to the A-loop region that is complementary to the anticodon region of $tRNA^{Lys,3}$. The 18-nucleotide (bold sequence) 3′ to the A-loop region is the primer binding site (PBS), which is complementary to $tRNA^{Lys,3}$ for NL-4-WT and NL-4-WT (G490E). These viruses differ by a mutation in the RT gene (G to A nucleotide 4018 of the NL-4 sequence), which results in a G490E mutation. Proviral constructs in which the PBS and A-loop region/PBS were mutated to correspond to $tRNA^{Trp}$ were taken from a previous study (Kang et al., 1996). The region complementary to the anticodon region of $tRNA^{Trp}$ is denoted in bold (TCTGGAGT) and the PBS downstream of this region also noted in bold (NL-4-Trp; NL-4-Trp-AC). The G490E mutation was engineered into proviruses in which the PBS and PBS with A-loop were mutated to be complementary to $tRNA^{Trp}$ resulting in the notation G490E (NL-4-Trp (G490E); NL-4-Trp-AC (G490E)). (B) Infectivity of wild-type and mutant viruses from transfection of 293T cells. Proviral DNA was transfected into 293T cells, and the amount of infectious virus in the supernatant was determined using the JC53 assay (Derdeyn et al., 2000). The infectivity represents the amounts of infectious units divided by the levels of p24 antigen. The level of infectivity obtained from the wild-type virus (NL-4-WT) was set at 100%. The amounts of infectious virus obtained from the wild-type virus containing the G490E mutation in RT as well as the viruses with a PBS and PBS A-loop complementary to $tRNA^{Trp}$ containing the G490E mutation were also determined. The levels of p24 were determined by ELISA. Standard deviations are represented by the error bars.

type (NL-4) virus was set at 100% with the infectivities of the mutants compared to this level (Fig. 1B). The G490E mutation in the wild-type virus reduced the infectivity of the wild-type virus by approximately 60% in this assay. Viruses in which the PBS alone or PBS plus A-loop were mutated to be complementary to $tRNA^{Trp}$ demonstrated a reduced infectivity compared to the wild type. For the virus with only the PBS complementary to $tRNA^{Trp}$, we observed an infectivity that was approximately 10% of that of the wild-type level, while for viruses with both PBS and A-loop, the infectivity was only 3% of the wild-type levels. Consistent with the results obtained with the wild-type virus, the G490E mutation in RT also reduced the infectivity of the viruses with a PBS complementary to $tRNA^{Trp}$. In contrast, infectivities of virus with both the PBS and A-loop complementary to $tRNA^{Trp}$ had similar infectivities (albeit very low), as the virus with the wild-type RT.

We next examined the infectivity of these viruses following continuous culture with SupT1 cells (Fig. 2A). We initiated the infections with equal amounts of infectious virus and followed the p24 levels in the cultured supernatant for approximately 35 days. The wild-type virus demonstrated a rise in p24 levels peaking at approximately day 14 post-initiation of culture. There were no clear-cut differences between the levels of p24 observed in cultures from the wild-type viruses with or without the G490E mutation in RT. In contrast, the viruses with the PBS only altered to be complementary to $tRNA^{Trp}$ demonstrated a delay in replication. By day 14 of culture, viruses with the wild-type RT began to increase, peaking at day 28 of culture. The

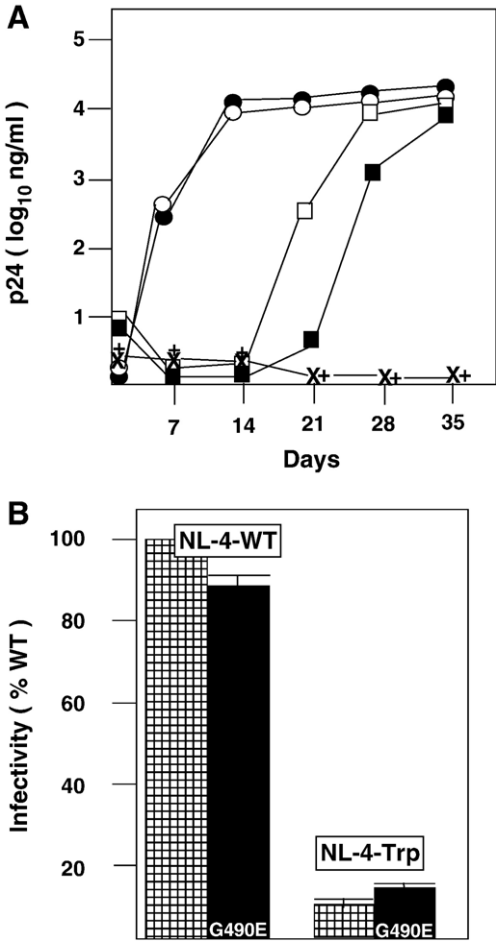

Fig. 2. Replication of viruses in SupT1 cells. (A) Infections were initiated in SupT1 cells as described in the Materials and methods. Virus replication was monitored by the levels of p24 antigen in the culture supernatants selected at days following initiation of culture. The key is as follows: solid circles, NL-4-WT; open circles, NL-4-WT (G490E); open box, NL-4-Trp; closed box, NL-4-Trp (G490E); X, NL-4-Trp-AC; + NL-4-Trp-AC (G490E). Both proviruses containing the Trp-AC mutations did not have p24 antigen in the culture supernatants over the background value obtained from mock transfection. The data are representative of three independent virus infections. (B) The infectivity of virus obtained following day 35 of culture in SupT1 was determined using the JC53 assay. Infectivity represents the infectious units divided by the p24 antigen obtained from the cultured supernatant at day 35 post-infection as shown from panel A. Note that the viruses with the anticodon mutation complementary to Trp did not grow and therefore were not included in this assay. Virus obtained from NL-4-WT was set at 100%. The levels of NL-4-WT (G490E) and NL-4-Trp with and without the G490E are presented. Error bars are standard deviation.

viruses with the G490E mutation in RT were delayed but also began to increase approximately 7 days after (day 21 of culture), then increased to a level similar to that of the wild-type viruses by day 35. In contrast, viruses in which both the PBS and A-loop were mutated to be complementary to $tRNA^{Trp}$ did not grow in SupT1 under these culture conditions; regardless of whether the RT was mutated, both viruses did not show any increase in p24 following long-term in vitro culture.

One of the interesting features of the viruses in which the PBS is complementary to $tRNA^{Trp}$ is the propensity to revert to utilize $tRNA^{Met}$ or $tRNA^{Lys,3}$ (Kang and Morrow, 1999; Kang et al., 1996). In a previous study, we had identified viruses in which both the PBS and A-loop complementary to $tRNA^{Trp}$ resulted in the virus to mutate to utilize $tRNA^{Met}$. Since only the viruses with the PBS complementary to $tRNA^{Trp}$ grew in SupT1 cells, we analyzed the high molecular weight DNA from these viruses to determine if the PBS was stable. Analysis of the cultures derived from day 35 infection in SupT1 cells revealed that these viruses had reverted to utilize $tRNA^{Met}$, regardless of whether or not the RT mutation was present (Table 1). Consistent then with our previous studies, we found that once the viruses had reverted to utilize $tRNA^{Met}$, they grew to levels in our culture system consistent with that observed for the wild-type virus. Interestingly, we have analyzed the infectivity of viruses obtained after day 35 cultured in SupT1 cells (Fig. 2B). This time, we found that the wild-type virus with the G490E mutation was approximately 90% as infectious as the wild type which differs from the infectivity of this virus derived from transfection of 293 T cells. The reason for this difference is unclear but could be due to the source of the virus (293 T cells compared to SupT1 cells). Sequence analysis of the RT gene confirmed though that the viruses still had maintained the G490E mutation with no additional RT mutations (data not shown). In contrast, viruses in which the PBS was complementary to $tRNA^{Trp}$ (initially and following mutation to be complementary to $tRNA^{Met}$) grew at approximately 10% the level of the wild-type virus. Thus, although the assays for p24 production show that the viruses are growing in a similar fashion as the wild-type virus at day 35, the infectivity is still relatively low compared to the wild-type virus as measured in the JC53 assay. Collectively, the results of these studies establish that the replication of the viruses in which the PBS was complementary to $tRNA^{Trp}$ was not influenced by the G490E mutation in HIV-1 RT. Furthermore, the propensity to revert to utilize an alternative tRNA ($tRNA^{Met}$) or the further selection to generate a virus that uses $tRNA^{Lys1,2}$ was also not influenced by the RT mutation.

We next examined the infectivity of the viruses in PBMC. Again, the wild-type viruses with or without the G490E mutation in RT demonstrated substantial infectivity that peaked between days 7 and 14 post-initiation of the culture. There were no differences between the replication of the wild type containing the RT mutation. In contrast, viruses in which the PBS was altered to be complementary to $tRNA^{Trp}$ did not grow (or grew very poorly) following 35 days of in vitro culture, regardless of whether or not the viruses contained the mutation

Table 1
DNA sequence of PBS following extended cultures in SupT1 (Day 35)

Input	U5-PBS	RT
NL-4-Trp[a]	PBS region is complementary to $tRNA^{Met}$ [b]	No change[c]
NL-4-Trp(G490E)	PBS region is complementary to $tRNA^{Met}$	No change
NL-4-Trp-AC	No growth[d]	No growth
NL-4-Trp-AC(G490E)	No growth[d]	No growth

[a] Input proviral DNA.
[b] The PBS from integrated proviruses complementary to $tRNA^{Met}$.
[c] No change in WT or G490E mutation of RT from input.
[d] This virus did not grow; no PCR product recovered.

in RT (data not shown). The p24 values from these viruses did not increase over the culture period; similar to what we found with the viruses which contained both the A-loop and PBS complementary to $tRNA^{Trp}$, these viruses also did not grow during this culture period.

The results of these studies suggest then that the mutation in RT did not effect the replication of the wild-type virus in either SupT1 cells or PBMC. Furthermore, the replication of viruses in which the PBS was complementary to $tRNA^{Trp}$ alone was slightly delayed in SupT1 cells as a result of the 490E mutation. However, regardless of RT mutation, viruses contained both the anticodon and PBS complementary to $tRNA^{Trp}$ were non-infectious as determined by the infectivity assay from transfected cell culture supernatants as from analysis of growth in SupT1 and PBMC cultures.

Effect of G490E mutation in RT on virus replication that uses $tRNA^{Lys1,2}$ for reverse transcription

In a previous study, we have identified and characterized an HIV-1 in which it utilized $tRNA^{Lys1,2}$ rather than $tRNA^{Lys,3}$ for replication (Kang and Morrow, 1999; Kang et al., 1999). Interestingly, this virus was identified from the characterization of the long-term replication of the virus that uses $tRNA^{Met}$ for replication. We found that viruses could be stabilized to utilize $tRNA^{Lys1,2}$ if additional mutations were made within the A-loop region to be complementary to the anticodon of $tRNA^{Lys1,2}$. A recent study also characterized an HIV-1 which utilizes $tRNA^{Lys1,2}$ for replication (Abbink et al., 2004). In this case, additional mutations, made upstream of the A-loop region at a position identified as the primer activation signal, were required to be complementary to $tRNA^{Lys1,2}$ in order for the virus to stably utilize this tRNA for replication. During the characterization of this virus, the G490E mutation in RT was identified and postulated to co-operatively help the virus to maintain the use of $tRNA^{Lys1,2}$. Since the G490E mutation in RT did not impact on the stability or selection of tRNAs with viruses in which the PBS was altered to be complementary to $tRNA^{Trp}$, we wanted to determine whether or not this would be specific for the virus that uses $tRNA^{Lys1,2}$ as a result of A-loop modifications. In these studies, we made use of our previously described mutant in which both the A-loop and PBS were mutated to be complementary to $tRNA^{Lys1,2}$ (Fig. 3A). This region was cloned into the NL-4 provirus and the infectivity as well as replication

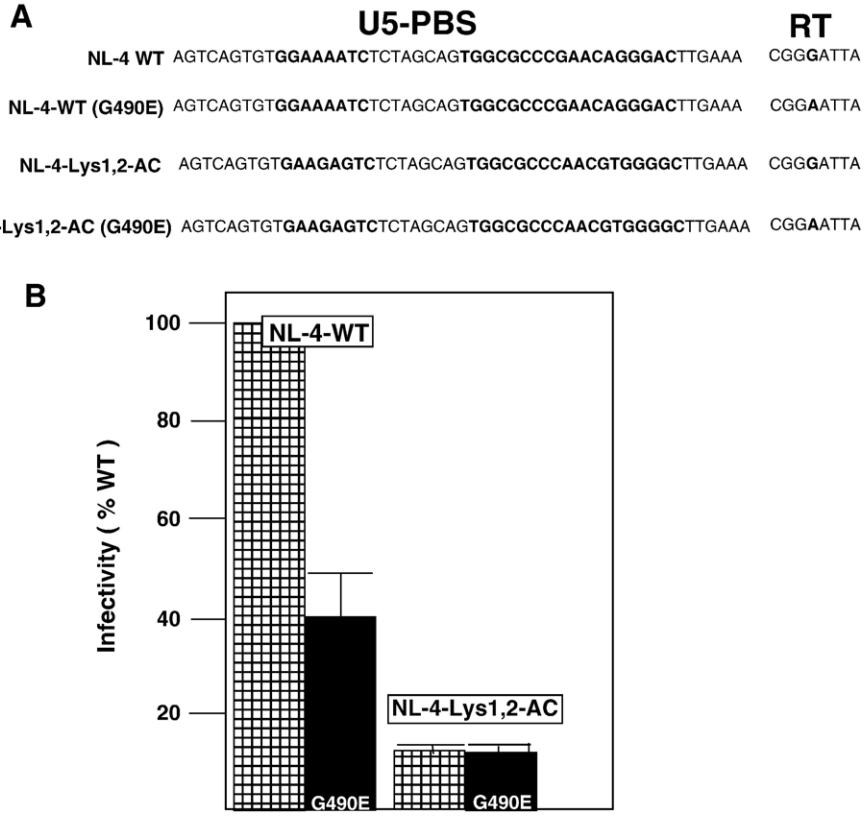

Fig. 3. Characterization of HIV-1 with PBS and A-loop complementary to $tRNA^{Lys1,2}$. (A) Sequence of HIV-1 with U5-PBS complementary to $tRNA^{Lys1,2}$. Proviral constructs in which the A-loop and PBS complementary to $tRNA^{Lys,3}$ (NL-4-WT) with and without mutations in the RT (G490E) are depicted as in Fig. 1A. A proviral construct NL-4-Lys12-AC in which the A-loop and PBS are complementary to $tRNA^{Lys1,2}$ (shown in bold) was taken from a previous study (Moore-Rigdon et al., 2005). Mutations in the RT gene (G490E) were constructed into the proviral clone to give NL-4-Lys12-AC (G490E). (B) Infectivity of wild type and HIV with PBS and A-loop complementary to $tRNA^{Lys1,2}$. The infectivities of wild type and NL-4-Lys12-AC and NL-4-Lys12-AC (G490E) were determined following transfection of proviral constructs into the 293T cells. The amount of infectious units obtained in the supernatants was determined by the JC53 assay; the levels of p24 antigens were measured by ELISA. Infectivity for the wild type (IUs/p24) was set at 100%. The values for the wild type with the G490E mutation (NL-4-WT (G490E)) and viruses in which the PBS and A-loop were mutated to be complementary to $tRNA^{Lys1,2}$ (NL-4-Lys12-AC) with and without the G490E mutation were determined. The value for wild type was set at 100%. The bars indicate standard deviation.

in SupT1 and PBMC was determined. We first analyzed the infectivity of viruses obtained following transfection in 293 T cells. As presented previously, we found that wild-type viruses, which contained the G490E mutation in RT, had approximately 40% the infectivity of wild-type viruses. Virus which contained the A-loop and PBS mutated to be complementary to the $tRNA^{Lys1,2}$ had infectivities that were approximately 10% of that of the wild-type virus (regardless of whether or not the RT mutation was present) (Fig. 3B).

We first analyzed the replication of these viruses in SupT1 cells (Fig. 4A). Cultures were initiated with similar amounts of infectious units, and the amounts of p24 produced in the culture supernatant were analyzed over a 35-day culture period. As shown previously for the wild type, the presence or absence of the G490E mutation in RT did not significantly impact on the replication of the virus in SupT1 cells. We found a rise in p24 production starting at day 7 that peaked by 14 days post-initiation of culture and was maintained for the 35-day culture period. Interestingly, similar results were found with viruses in which the PBS and A-loop were altered to be complementary to $tRNA^{Lys1,2}$. In this case, we noted a slight delay in the production of p24 in the culture supernatant at day 7 compared to the wild-type levels, but by day 14, these levels had reached a peak value that was similar to that for the wild-type virus. There was no difference with respect to the amounts of p24 produced in the cultures where viruses contained the 490E mutation in RT. We next characterized the replication of these viruses in PBMC (Fig. 4B). Again, for these studies, we initiated the cultures with similar amounts of IUs and followed the replication by the presence of p24 in the culture supernatant. There was no significant difference between the replication of the wild-type virus with or without the G490E mutation. In contrast, viruses in which the PBS and anticodon were complementary to $tRNA^{Lys1,2}$ grew poorly in PBMC, reaching levels approximately 1% of that of the wild-type virus by day 14 and day 21 post-initiation of culture. The levels of p24 were significantly higher than that of the background levels (or levels obtained from viruses which contained the PBS complementary to $tRNA^{Trp}$) indicating that replication had occurred. We next analyzed the U5-PBS from viruses which were complementary to $tRNA^{Lys1,2}$. Consistent with our previous studies, we found that these viruses maintained a PBS complementary to $tRNA^{Lys1,2}$ (data not shown). There is no difference between viruses which contained the G490E mutation and the wild-type RT. Furthermore, sequence analysis of the RT gene revealed that the mutation was stable following in vitro culture in PBMC.

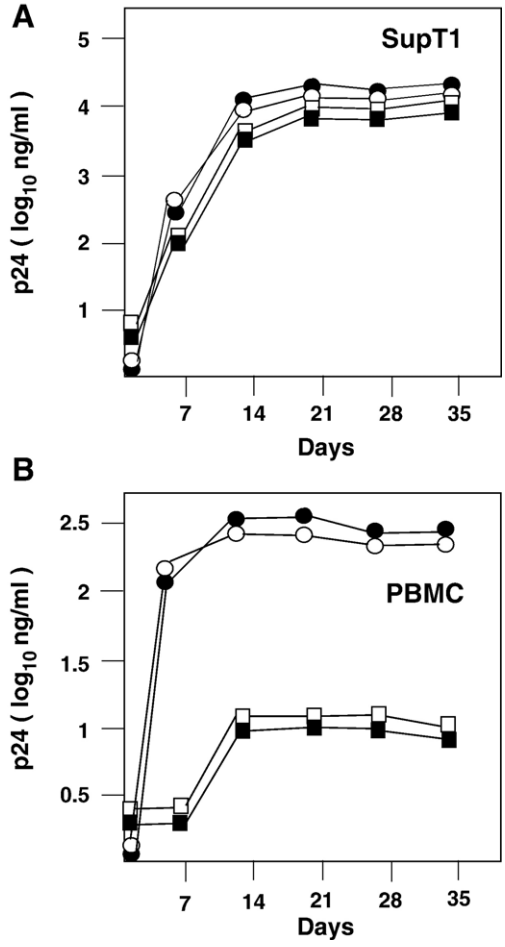

Fig. 4. Replication of HIV-1 with U5-PBS complementary to $tRNA^{Lys1,2}$ containing the G490E mutation. (A) SupT1 cells were infected with NL-4 wild type (with and without G490E mutation) and NL-4-Lys12-AC (with and without G490E mutation). Infection was monitored by assaying levels of p24 antigen in the culture supernatants at selected days for 35 days. (B) PBMC. Infection of the NL-4-WT (with and without G490E mutation) and NL-4-Lys12-AC (with and without G490E mutation) was established in PBMC. Infection was monitored by levels of p24 antigen in the culture supernatant at selected days for 35 days. Key to both panels: closed circles, NL-4-WT; open circles, NL-4-WT (G490E); closed square, NL-4-Lys12-AC; open square, NL-4-Lys12-AC (G490E). The data presented are representative from three independent infections.

Given that the viruses were stable following culture in PBMC, we wanted to determine if there was a subtle effect of the G490E mutation that would allow a greater fitness in culture. For these studies, we initiated cultures with varying input ratios of wild type to G490E mutated viruses (Table 2). As a control, we first analyzed the wild-type virus replication using input ratios of 10:1 (wild type) RT to G490E mutation, 1:1 and 1:10, respectively. In cases where the dominant virus that initiated cultures was either the wild-type RT or the mutant RT, we found that these viruses still dominated following 35 days of culture in PBMC. Thus, at a 10:1 ratio of either virus, there was no significant advantage of the wild type over the mutant RT. Initiating cultures with equal amounts of infectious units followed by analysis of the RT revealed a similar equal distribution following TA cloning of PCR amplified RT genes from these cultures at day 35. In this case, 10 of the 18 TA clones obtained were from the wild-type RT as the remaining 8 were from the G490E mutant. We next compared the fitness of viruses in which both the A-loop and PBS were complementary to $tRNA^{Lys1,2}$. Using the same strategy for analysis, we found that again if the dominant virus contained either the wild-type or mutant RT, we recovered mainly this RT following in vitro culture of 35 days with the PBMC. Initiating cultures with equal amounts of virus followed by TA cloning of the amplified RT genes after 35 days of culture in PBMC revealed a slight advantage for the RT G490E mutant compared to the wild type (13 of 19 TA clones, compared to 6 of 19 TA clones). This result

Table 2
Replication competition experiments between HIV with WT or G490E mutation in RT

Virus[a]	Input[b] RT-WT:RT-G3600A	Sample[c]	Sequencing of RT Region
NL-4	10 to 1	PCR	RT-WT
NL-4	1 to 1	TA cloning	10/18 RT-WT, 8/18 RT-G490E
NL-4	1 to 10	PCR	RT-G490E
NL-4-Lys1,2-AC	10 to 1	PCR	RT-WT
NL-4-Lys1,2-AC	1 to 1	TA cloning	6/19 RT-WT, 13/19 RT-G490E
NL-4-Lys1,2-AC	1 to 10	PCR	RT-G490E

[a] Virus used in competition (NL-4-WT or NL-4-Lys1,2-AC).
[b] Starting ratio of virus in infectious units as determined by JC53-BL assay.
[c] Sample used for DNA sequencing. PCR refers to direct sequencing of PCR product for high molecular weight DNA. No ambiguities in the RT region encompassing G490E. TA cloning refers to PCR product cloned into TA vector and individual colonies sequenced.

suggests there could be a fitness advantage for viruses which contain the G490E mutation in RT that utilized $tRNA^{Lys1,2}$ for initiation of reverse transcription. However, the dominant effect for viruses to utilize $tRNA^{Lys1,2}$ or other alternative tRNAs appears to reside in the PBS and U5 region rather than the RT mutation.

Discussion

In the current study, we have investigated the potential of a mutation in the HIV-1 RT (G490E) to influence primer selection. This mutation in RT was previously identified to facilitate the use of $tRNA^{Lys1,2}$ as a primer for HIV-1 reverse transcription (Abbink et al., 2004). We found that viruses in which the PBS alone or PBS and A-loop were mutated to be complementary to $tRNA^{Trp}$ were not influenced by the presence or absence of the G490E mutation in RT. Regardless of whether the RT mutation was present, the virus with a PBS complementary to $tRNA^{Trp}$ mutated to use $tRNA^{Met}$. We next examined the effects of the G490E mutation on the replication of HIV-1 in which the A-loop and PBS had been altered to be complementary to $tRNA^{Lys1,2}$. Previous studies from our laboratory had demonstrated that this virus stably maintains a PBS complementary to $tRNA^{Lys1,2}$ (Kang and Morrow, 1999, Kang et al., 1999). Comparison of the replication of viruses with and without the G490E mutation revealed no substantial differences. Thus, while the G490E mutation in RT might facilitate the replication of HIV-1 with a PBS and upstream region in the U5 (PAS) complementary to $tRNA^{Lys1,2}$, this RT mutation does not affect the replication of viruses with mutations in the A-loop region of U5 that facilitate the use of alternative tRNA primers.

Previous studies from our laboratory and others have used genetic manipulation of the HIV-1 genome to gain insights into the mechanism by which HIV-1 selects the primer tRNA required for HIV-1 replication. We and others have shown that alteration of the PBS so as to be complementary to alternative tRNAs results in viruses that utilize these tRNAs for short-term in vitro replication but rapidly revert back to utilize $tRNA^{Lys,3}$ (Das et al., 1995; Jiang et al., 1994; Wakefield et al., 1995). To stabilize the use of these alternative tRNAs, we have made additional changes within the U5 region that are complementary to the anticodon region of certain tRNAs. This strategy has allowed us to derive HIV-1 which can stably utilize $tRNA^{Met}$, $tRNA^{Lys1,2}$, $tRNA^{His}$ and more recently $tRNA^{Glu}$ (Dupuy et al., 2003; Kang and Morrow, 1999, Kang et al., 1997, 1999; Wakefield et al., 1996; Zhang et al., 1998). Not all U5-PBS combinations allow the virus to utilize alternative tRNAs such that viruses with A-Loop-PBS combinations complementary to $tRNA^{Ile}$, $tRNA^{Pro}$, and $tRNA^{Trp}$ were not stable and reverted back to utilize $tRNA^{Lys,3}$ or in some instances ($tRNA^{Trp}$), alternative tRNAs. In a previous study, we characterized the virus that utilizes $tRNA^{His}$ for replication. From DNA sequence analysis of integrated proviruses, we found that there were no consistent mutations in the structural genes (nucleocapsid and RT), suggesting that mutations did not occur to compensate for the use of alternative tRNAs (Zhang et al., 1996, 1998). In support of this, we noted that the virus could rapidly utilize $tRNA^{His}$ following mutation of the A-loop and PBS, suggesting that no additional mutations in structural genes were required. It is important to note though that these viruses, as well as all of our viruses which utilize alternative tRNAs for replication, do not replicate with the same efficiency as the wild-type HIV-1. Similar to what we have presented in this study, we noted that for the most part, viruses in which the PBS or PBS and A-loop had been altered, exhibit approximately 1/10 the infectivity as that for the wild-type HIV-1. Thus, the recent study by Abbink et al. was of particular interest because the authors have shown that HIV-1 can be forced to utilize $tRNA^{Lys1,2}$ following mutations within the PBS and U5 (PAS, not A-loop region) (Abbink et al., 2004). Characterization of this virus revealed an additional mutation in the RT gene, G490E, which facilitated the replication of HIV-1 with a PBS complementary to $tRNA^{Lys1,2}$. The basis of our experiments then was to test whether or not this mutation in HIV-1 RT could impact on viruses which utilized other tRNAs ($tRNA^{Trp}$) as well as a virus in which we had described that utilizes $tRNA^{Lys1,2}$ but contains a mutation within the A-loop rather than the PAS. The results of our studies clearly show that the G490E mutation does not substantially effect the replication or the primer utilization of the viruses in our study that have been engineered to be complementary to $tRNA^{Trp}$ or $tRNA^{Lys1,2}$. Interestingly, the virus which was engineered to be complementary to $tRNA^{Trp}$ has, in previous studies, been shown to mutate in culture to utilize $tRNA^{Met}$ or $tRNA^{Lys1,2}$ under the appropriate conditions (Kang et al., 1996). If the HIV-1 RT G490E mutation facilitated the use of $tRNA^{Lys1,2}$, we would have expected the virus to utilize $tRNA^{Trp}$ to mutate directly to utilize $tRNA^{Lys1,2}$ or during the culture period revert to selectively utilize $tRNA^{Lys1,2}$. Clearly, this was not observed in our studies. Thus, we believe that the G490E mutation in RT, while it can contribute some to the increased HIV-1 replication for the virus with the PBS complementary to $tRNA^{Lys1,2}$, could only be manifested in the viral competition experiments between isogenic genomes that only contain the G490E

mutation (Table 2). We did not see any changes in the preference of viruses that were engineered to utilize $tRNA^{Trp}$ to select $tRNA^{Lys1,2}$.

The results of our studies and Abbink et al. support the concept that the U5-PBS region is the major determinant for the selection of the tRNA primer, and that HIV-1 can be forced to utilize alternative primers through mutations in the U5 and PBS. Alteration of the PBS with either the A-loop or PAS can result in HIV-1 stably utilizing $tRNA^{Lys1,2}$ for replication. However, in each case, the virus does not grow as well as the wild-type virus. This result is interesting when in the context that $tRNA^{Lys1,2}$ is incorporated into the HIV-1 genome at levels similar to and many times greater than that for the preferred tRNA primer, $tRNA^{Lys,3}$ (Jiang et al., 1993). If primer selection relied merely on just the position of the tRNA primer in close proximity to the PBS, one would suspect that alteration of the PBS and A-loop/PAS to be complementary to $tRNA^{Lys1,2}$ should result in a virus that has replication characteristics similar to that of the wild-type virus. Indeed, slight mutations in RT to accommodate the differences between $tRNA^{Lys,3}$ and $tRNA^{Lys1,2}$ (such as the G490E mutation) would be expected to occur when the virus was forced to utilize $tRNA^{Lys1,2}$ that would lead to viruses with replication characteristics similar to that of the wild-type virus. The fact that all viruses in which the PBS has been altered to be complementary to alternative tRNAs have replication characteristics that differ from the wild-type virus suggests that primer selection might be coupled with other elements of the viral replication cycle. Previous studies from this laboratory have suggested that primer selection is coupled with translation in that tRNAs which are aminoacylated are most effectively selected as primers for HIV-1 reverse transcription (Kelly and Morrow, 2003; Kelly et al., 2003). These results lead to the suggestion that HIV-1 primer selection could possibly occur in conjunction with HIV-1 translation and/or the switch from translation to encapsidation of the viral genomic RNA. With respect to this issue, it is possible that the mutations required in the U5 region (PAS and/or A-loop) act in a similar fashion to reduce the replication of HIV-1 to facilitate the selection of alternative tRNAs. Consequently, this implies that HIV-1 selects the tRNA primer from a pool of intracellular tRNAs that is highly enriched with $tRNA^{Lys,3}$ to facilitate high-level replication. Alteration of the U5-PBS region results in a virus that replicates slower, possibly because the virus required tRNAs that are not dominant in the intracellular pool (e.g., $tRNA^{Lys1,2}$). Additional experiments with mutations in the U5 region (PAS and A-loop) would be required to substantiate this possibility and further define the mechanism of primer selection.

Materials and methods

Tissue culture

The SupT1 cells were maintained in RPMI 1640, supplemented with 15% fetal bovine serum (FBS) and 1% antibiotic–antimycotic. The 293 T cells were maintained in DMEM plus 10% FBS and 1% antibiotic–antimycotic. Human peripheral blood mononuclear cells (PBMC) were collected, stimulated using rIL-2 and phytohemagglutinin (PHA) (Sigma, St. Louis, MO), and maintained as described previously (Dupuy et al., 2003). All the cell culture media were from Cellgro by Mediatech, Herndon, VA.

Construction of NL4-3 proviruses containing PBS and RT mutations

The NL-4-Lys1,2-AC mutant was described as previously (Moore-Rigdon et al., 2005). For the construction of NL-4-Trp and NL-4-Trp-AC mutants, proviral clones pHXB2-Trp and Trp-AC (Kang et al., 1996) were used to provide the U5-PBS region. After digestion with *Hpa*I and *Bss*HII restriction enzymes, an 868-bp fragment that contained the 5′ LTR, PBS, and leader region *gag* of HXB2 was separated and cloned into the NL4-3 HIV-1 proviral plasmid using *Hpa*I and *Bss*HII restriction sites. All resulting NL4-3 constructs were verified by DNA sequencing.

To construct the RT mutation, the RT region of NL-4 PBS mutants was cloned into Litmus28 vector after digestion by *Age*I and *Eco*RI. QuickChange site-directed mutagenesis of RT region was carried out on Litmus28 using PCR methods; template DNA (400 ng), *pfu* Turbo DNA polymerase (Stratagene), Turbo DNA polymerase buffer, and dNTPs were used for PCR as described by the manufacturer, PCR was performed by three cycling (95 °C for 30 s, 55 °C for 1 min, and 68 °C for 5 min) for 16 cycles, then elongation for 10 min at 68 °C. The synthetic oligonucleotides primers for mutant RT were 5′-TCTAGCTTTGCAGGATTCGGAATTAGAAG-TAAACATAGTGAC-3′ (sense) and 5′-GTCACTATGTT-TACTTCTAATTCCGAATCCTGCAAAGCTAGA-3′ (anti-sense). The underlined nucleotides are the ones changed from the wild type. After PCR the methylated parental DNA template was digested with *Dpn*I for 1 h at 37 °C, and then the reaction mixture containing DNA was transformed into DH5-α cells. Colonies were selected on LB agar plates with ampicillin (100 μg/ml). The mutations were confirmed by DNA sequencing. Finally, the segment of RT region was separated from Litmus28 after digestion by *Age*I and *Eco*RI and ligated to NL-4 that had been digested by *Age*I and *Eco*RI.

DNA transfections

Transfections were performed according to the protocol for the Fugene 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 2 μg of proviral plasmid DNA and 6 μl Fugene reagent were added to 200 μl of DMEM (no FBS). This mixture was incubated at room temperature for approximately 30 min then added drop-wise to one well of a 6-well plate containing 50% confluent 293 T cells in DMEM with 10% FBS. The transfections were incubated overnight at 37 °C, then the medium was replaced with fresh DMEM containing 10% FBS. After 48 h, all supernatants were collected and filtered through 0.45-μm-pore-size syringe filters and stored at −80 °C. Supernatants from transfected cells were assayed for HIV-1 p24 antigen by

ELISA (Beckman Coulter, Miami, FL). Infectivity was also determined by JC53BL assay as described previously (Moore-Rigdon et al., 2005).

Infection of SupT1 and PBMC

Infections were performed by inoculating 1×10^6 SupT1 cells or 5×10^6 PHA-stimulated PBMC with viral supernatant containing 1000 IU of virus. Following incubation for 2 h at 37 °C, with shaking every 30 min, virus/PBMC mixtures were transferred to 25-cm^2 tissue culture flasks, and the final volumes were adjusted to 10 ml with RPMI 1640, containing 15% FBS. PBMC were also cultured with 30 U/ml rIL-2.

Every 3 days, the infected SupT1 cells were passaged 1:5. For PBMC cultures, half of medium was replaced every 3 days without removing PBMC. Every 7 days, 1 ml of cell suspension was removed for SupT1 and PBMC cultures and centrifuged at 24,000 × *g* for 2 min. Supernatant was separated from the cell pellets and stored at −80 °C for further analysis by p24 ELISA and JC53BL infectivity assays. Cell pellets were also stored at −80 °C for isolation of high molecular weight DNA. Once the SupT1 cell culture was cleared of cells, 1×10^6 SupT1 cells were added to the culture every 3 days. Every 14 days, an additional 5×10^6 PHA-stimulated PBMC were added to PBMC culture.

Dual infection assay

Virus with wild-type RT or mutant RT was mixed with the ratio of wild-type RT to mutant RT as follows: A: 10 IU to 1000 IU; B: 1000 IU to 1000 IU; and C: 1000 IU to 10 IU. SupT1 and PBMC cells were infected by different mixtures as described in the previous section. At day 35, the cell pellets were collected for PCR and DNA sequence analysis.

Subcloning of PCR products and DNA sequencing

High molecular weight DNA was extracted from infected SupT1 or PBMC cell pellets following the instructions of Wizard Genomic DNA Isolation Kit (Promega). The regions of U5-PBS and RT were amplified by PCR from the high molecular weight DNA. The primers used to amplify U5-PBS regions are 5′-CGGAATTCTCTCCTTCTAGCCTCCGCTAGTC-3′ and 5′-CCTTGAGCATGCGATCTACCACACACAAGGC-3′. The primers used to amplify RT regions are 5′-GACAGAG-TATTGGCAAGCCACCTGGAT-3′ and 5′-GCCA-TATTCGTGGGCTACAGTCTACTT-3′. The resulting PCR products were gel purified and ligated to the pGEM-T Easy vector (Promega), and the ligation mixes were used to transform competent *Escherichia coli* DH5-α cells. Individual colonies were screened for DNA inserts, and the DNA sequence was determined by automated sequencing.

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