The bacterial tRNA\textsuperscript{Lys}-specific PrrC-anticodon nuclease efficiently cleaved an anticodon stem-loop (ASL) oligoribonucleotide containing the natural modified bases, suggesting this region harbors the specificity determinants. Assays of ASL analogs indicated that the 6-threonylcarbamoyladenosine modification (t\textsuperscript{6}A37) enhances the reactivity. The side chain of the modified wobble base 5-methoxycarbonylaminomethyl-2-thiouridine (mcm\textsuperscript{5}s\textsuperscript{2}U) has a weaker positive effect depending on the context of other modifications. The s\textsuperscript{2}U34 modification apparently has none and the pseudouridine (\textsuperscript{ψ}39) was inhibitory in most modification contexts. GC-rich but not IC-rich stems abolished the activity. Correlating the reported structural effects of the base modifications with their effects on anticodon nuclease activity suggests preference for substrates where the anticodon nucleosides assume a stacked A-RNA conformation and base pairing interactions in the stem are destabilized. Moreover, the proposal that PrrC residue Asp\textsuperscript{287} contacts mnm\textsuperscript{5}s\textsuperscript{2}U34 was reinforced by the observations that the mammalian tRNA\textsuperscript{Lys}-3 wobble base 5-methoxy carbamyl methyl-2-thiouridine (mcm\textsuperscript{5}s\textsuperscript{2}U) is inhibitory and that the D287H mutant favors tRNALys-3 over tRNALys-1. These determinants must include the anticodon sequence, in the anticodon loop and lower part of the anticodon stem (9). ACNase has suggested that the specificity determinants reside adjacent members of which (D287Y, S288P) alter the cleavage site specificity (10). Other mutations directed into this site (D287H, D287Q, or D287N) alter the specificity so that tRNA\textsuperscript{Lys}-3 forms with a hypomodified wobble base, otherwise poor substrates, are rendered more reactive than the natural. These compensatory effects suggest the existence of a specific interaction between the Asp\textsuperscript{287} residue of PrrC and the modified wobble base (11).

Comparing bacterial and mammalian tRNA\textsuperscript{Lys} substrates of ACNase has suggested that the specificity determinants reside in the anticodon loop and lower part of the anticodon stem (9). These determinants must include the anticodon sequence, judged from the following observations. First, the anticodon of tRNA\textsuperscript{Lys} resembles the anticodons of most secondary substrates cleaved when PrrC is overexpressed (10). Second, most single anticodon base substitutions in unmodified tRNA\textsuperscript{Lys} abolish ACNase reactivity. Third, a substrate analog with a tRNA\textsuperscript{Lys}-3 anticodon transplanted in an otherwise tRNA\textsuperscript{Lys} sequence is as reactive as the wild type tRNA\textsuperscript{Lys} sequence (11).

The antibiotic action of the core polypeptide PrrC has not been purified to homogeneity. Yet PrrC certainly harbors the ACNase function, judged from the manifestation of ACNase activity by E. coli (8) and mammalian cells overexpressing PrrC (9). Moreover, a tRNA\textsuperscript{Lys}-3 anticodon recognition site has been pinpointed in a cluster of selected PrrC mutations, two adjacent members of which (D287Y, S288P) alter the cleavage site specificity (10). Other mutations directed into this site (D287H, D287Q, or D287N) alter the specificity so that tRNA\textsuperscript{Lys}-3 forms with a hypomodified wobble base, otherwise poor substrates, are rendered more reactive than the natural. These compensatory effects suggest the existence of a specific interaction between the Asp\textsuperscript{287} residue of PrrC and the modified wobble base (11).

A tRNA\textsuperscript{Lys}-specific anticodon nuclease (ACNase)\textsuperscript{1} in latent form is encoded by the optional Escherichia coli prr locus (1). It comprises the core ACNase polypeptide PrrC and type IC DNA restriction-modification enzyme EcoPrrI that masks PrrCs activity (2–5). The phage T4-coded peptide Stp inhibits EcoPrrI DNA restriction and activates ACNase (6). Cleavage of tRNA\textsuperscript{Lys} 5’ to the wobble base enaues, yielding 2’:3’-cyclic phosphate (2’:3’-%p> and 5’-OH termini. However, the lesion is normally offset by the phage T4-encoded polynucleotide kinase and RNA ligase (7).
These results indicate that the tRNA acceptor domain helps position ACNase for the native substrate and that alternative RNA-protein interactions may form in the ACCA truncated mutants as well as for A73 mutants.

Here we report that ACNase efficiently cleaves a synthetic tRNA<sup>1</sup><sup>-</sup>ASL containing the base modifications of <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>ASLs. Comparing it with various hypomodified and unmodified analogs revealed opposing effects of the base modifications on ACNase reactivity. Correlating these data with the observed contributions of the base modifications to the ASL solution structure (12, 14, 15) leads us to suggest that ACNase prefers substrates where the anticodon nucleotides assume a stacked A-RNA conformation and where the base pairing interactions in the ASL stem are relatively destabilized. We also show that the PrrC D287H mutation renders human tRNA<sup>1</sup><sup>-</sup>ASLs relatively more reactive than the natural substrate. This finding and the inhibitory effect of the side chain of the tRNA<sup>1</sup><sup>-</sup>ASL modified wobble base (5-methoxycarbonylmethyl-2-thiouridine, mc<sup>5</sup>ms<sup>2</sup>U34) reinforce the previously proposed interaction between PrrC residue Asp<sup>287</sup> and the side chain of the <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>ASL wobble base (11). This result and the ability to cleave isolated ASLs raise the interesting prospect of directing ACNase against tRNA<sup>1</sup><sup>-</sup>ASLs annealed to the genomic RNA template in the priming complex of the human immunodeficiency virus.

**EXPERIMENTAL PROCEDURES**

**Materials**—All ASL oligonucleotides used in this study are listed in Table I. The ASL oligonucleotides 1–15 in Table I were chemically synthesized. The methods have been described for oligonucleotides 1, 3, 4, 6, and 7 (13) oligonucleotides 14 and 15 (16) and the syntheses of oligonucleotides 8–11 will be described elsewhere. 2 ASL oligonucleotides 16–23 were transcribed in vitro using T7 RNA polymerase according to an established procedure (17). Those containing inosine (ASLs 20–22) were transcribed using GMP as primer and ITP instead of GTP. The in vitro transcribed ASLs were dephosphorylated by calf intestinal alkaline phosphatase. All ASLs were 5’-end labeled using T4 polynucleotide kinase.

**RESULTS**

**Specific and Efficient Cleavage of the Fully Modified tRNA<sup>1</sup><sup>-</sup>ASL by ACNase**—An ASL 17-mer containing the three modified bases of <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>ASLs (henceforth the reference ASL, Fig. 1), various hypomodified and unmodified derivatives and ASLs containing modified bases of mammalian tRNA<sup>1</sup><sup>-</sup>ASLs (Table I) were examined as ACNase substrates. The reference ASL and other chemically synthesized ASLs (ASLs 1–15) matched the mammalian tRNA<sup>1</sup><sup>-</sup>ASL sequence, which differs from that of <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>ASL in the upper three base pairs of the anticodon stem (compare the left and right panels in Fig. 1). However, the upper portion of the stem was considered nonessential for recognition by ACNase (9). The synthetic ASLs 5, 12, and 13 contained an extra 3’-dT residue since a labile linker was utilized for the chemical synthesis of the ASLs containing mc<sup>5</sup>U and ms<sup>2</sup>U. The unmodified ASL 15 featuring the mammalian tRNA<sup>1</sup><sup>-</sup>ASL sequence was also chemically synthesized. Unmodified ASLs 16–23 were transcribed in vitro and all of them contained a 5’ terminal G residue dictated by the constraints of transcription with T7 RNA polymerase (17). The in vitro transcribed ASL 16 corresponded in sequence to <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>ASL (Fig. 1b). ASL 19 resembled the <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>Val<sup>1</sup> ASL sequence (19) except for a reversed 5’ to 3’ terminal GC base pair. The DNA templates of ASL 19 and of two other ASLs with arbitrary GC-rich stem sequences (ASLs 17 and 18) were also transcribed into insine-rich versions (ASLs 20–22). ASL 23 was a mutant of <i>E. coli</i> tRNA<sup>1</sup><sup>-</sup>Ser in sequence in which A37 was replaced by G.

Incubation of the 5’-<sup>32</sup>P-labeled reference ASL with a partially purified ACNase preparation under conditions that minimize nonspecific degradation ("Experimental Procedures") yielded a heptanucleotide-like product missing from the ASL-negative control (Fig. 2, compare lanes 2 and 3 with lane 4). This product had a slightly reduced electrophoretic mobility compared with a heptanucleotide containing a 2’-3’-cyclic-phosphodiesterase and 3’-phosphatase activities (7, 20), shifted the product to the 3’ terminal phosphate within a marker ladder generated by partial alkaline hydrolysis of the reference ASL (Fig. 2, compare lanes 1 and 4). This retardation of the ACNase product could be accounted for by the lower negative charge of the 2’-3’-cyclic phosphate end-group expected to be found in the ACNase product. Incubating the ACNase product with T4 polynucleotide kinase, endowed with 2’-3’-cyclic-phosphodiesterase and 3’-phosphatase activities (7, 20), shifted the product to the position of the 2’-dephosphorylated heptanucleotide marker (compare lanes 5, 6), consistent with removal of the cyclic phosphate end group. Hence, ACNase appeared to cleave the ASL similar to the natural substrate, targeting the same site and producing the same cleavage termini.

Kinetic comparison of the reference ASL and full sized tRNA<sup>1</sup><sup>-</sup>ASL (Table II) yielded respective <i>K</i><sub>cat</sub> (for a PrrC-D222E hexamer) of −0.04 min<sup>−1</sup> and −0.02 min<sup>−1</sup> and <i>K</i><sub>m</sub> of 2.6 × 10<sup>−7</sup> M and 0.8 × 10<sup>−7</sup> M. A 2-fold faster cleavage of the ASL was also demonstrated by digesting the two substrates in the same reaction mixture (Fig. 3).

**Contributions of ASL Base Modifications to ACNase Reactivity**—In vitro transcribed, unmodified tRNA<sup>1</sup><sup>-</sup>ASL is less reactive...
The sequence of the reference ASL is derived from mammalian tRNA^Lys^3, which differs from *E. coli* tRNA^Lys^3 in the upper three bases of the anticodon stem. The mammalian tRNA^Lys^3 isoacceptor contains the modified wobble base 5-methylcytosine (m^5^C, m^5^s^2^U or U^9^), the modified base 3' to the anticodon is 2-methylthio-6-threonylcarbamoyladenosine (ms^2^t^6^A or A^9^), and the N-seryl pseudouridine (Ψ) stabilizes the A31-Ψ39 base pair. The single modified ASL resembles that of *E. coli* tRNA^Lys^3. The arbitrary GC-rich ASLs 1 and 2 are pGUUGACUUUAAUCAAC and pGCUGCUUUAAUACCGC, respectively. The Val^3^-like ASL resembles the *E. coli* tRNA^Lys^3 sequence except for reversal of the 5'-3' base pair. The IC-rich counterparts of the GC-rich and Val^3^-like ASLs (7.2 versus less than 0.2%, respectively). The negative effect of the mcm^5^s^2^U34 modification was further demonstrated by the different kinetic values of the ms^2^t^6^A37/Ψ39/Ψ39 side chain exerts a positive effect on ACNase reactivity. In contrast, the mcm^5^U34-ASL and mcm^5^s^2^U34-ASL, both containing the side chain of the *E. coli* Lys-3 wobble base, were less reactive than the corresponding unmodified ASL (Table I). Hence, the mcm^5^s^2^U34 and/or Ψ39 base modifications could attenuate ACNase reactivity of the reference ASL.

The specific contributions of the wobble base modifications to ACNase reactivity were evaluated similarly. The extent of cleavage of the mammalian ms^2^t^6^A37/Ψ39-ASL was 3-4-fold larger than that of the ss^2^U34/Ψ39-ASL (Table I). This suggested that the mammalian tRNA^Lys^3 side chain exerts a positive effect on ACNase reactivity. In contrast, the mcm^5^s^2^U34 and mcm^5^s^2^U34-ASL, both containing the side chain of the tRNA^Lys^3 wobble base, were less reactive than the corresponding unmodified ASL (Table I). The opposing effects of mammalian ms^2^t^6^A37 and mcm^5^s^2^U34 were underscored by the large difference in the extents of cleavage between the mammalian ms^2^t^6^A37/Ψ39 and mcm^5^s^2^U34/Ψ39 containing ASLs (7.2 versus less than 0.2%, respectively). The negative effect of the mcm^5^s^2^U34 modification was further demonstrated by the different kinetic values of the ms^2^t^6^A37/Ψ39/Ψ39-ASL (Table I). Hence, the mcm^5^s^2^U34 and/or Ψ39 base modifications could attenuate ACNase reactivity of the reference ASL.
Substrate Conformation Favoring by Anticodon Nuclease

The reaction mixtures (20 μl) contained 12 ng of PrrC (2.2 nM hexamer).

ψ39/3’-dT ASL ($K_m = 6.0 \pm 2.0 \times 10^{-8}$ M, $K_{cat} = 0.004$ min$^{-1}$).

Thus, mcm$^5$s$^2$U34 reduced the $K_{cat}$ by more than 3-fold and the binding affinity by 2-fold and the overall catalytic efficiency 7-fold. On the other hand, adding mcm$^5$s$^2$U34 to the t$^t$Aψ39 background increased $K_m$ by more than 3-fold and reduced the affinity 2-fold ($K_m = 2.6 \pm 0.6 \times 10^{-7}$ M, $K_{cat} = 0.044$ min$^{-1}$ versus $K_m = 1.7 \pm 0.7 \times 10^{-7}$ M, $K_{cat} = 0.144$ min$^{-1}$, respectively). Hence, the negative changes in catalytic efficiency conferred by mcm$^5$s$^2$U34 may be ascribed to the side chain rather than 2-thiol group. This inference is supported by the similar inhibitions caused by mcm$^5$s$^2$U34 and mcm$^5$s$^2$U34 when introduced into the unmodified background (Table I, compare ASLs 8 and 10 with 15). It is noteworthy that the lack of the s$^2$U34 modification from $E. coli$ tRNA$^{19s}$ had no detectable effect on the cleavage efficiency in vitro although in vivo this lesion was synthetically lethal with PrrC and severely inhibited tRNA$^{19s}$ cleavage (11).

The fully modified $E. coli$ tRNA$^{19s}$ mcm$^5$s$^2$U34/mcm$^5$s$^2$A37/ψ39-ASL was cleaved with a 10-fold higher $K_{cat}$ and exhibited a 3-4-fold weaker binding compared with mcm$^5$s$^2$U34/mcm$^5$s$^2$A37/ψ39/3’-dT-ASL carrying the base modifications of ψ39 into ASLs containing mcm$^5$s$^2$U34 or mcm$^5$s$^2$A37/mcm$^5$s$^2$A37/ψ39/3’-dT-ASL (Table I). However, confounding this comparison is the large negative effect of the extra 3’-dT residue in the latter substrate. Namely, against the mcm$^5$s$^2$U34/mcm$^5$s$^2$A37/ψ39 backdrop, the extra 3’-dT reduced the $K_m$ and $K_{cat}$ each by about an order of magnitude (Table II).

The presence of ψ39 instead of U39 rendered the ASL in certain cases less reactive. The unmodified ASL was cleaved to a 2-fold higher extent than the ψ39-containing counterpart. Similarly, introducing ψ39 into ASLs containing mcm$^5$s$^2$U34 or...
Mcms2U34 reduced their activity more than 5-fold (Table I). However, %39 had no apparent effect on either kinetic parameter when introduced in the mms2A373/-dT context and over the t6A37 background it even reduced the K_m < 3-fold (Table II).

**ASL Reactivity and Stem Stability—Unmodified ASLs with stems (underlined) corresponding in sequence to mammalian tRNA_{Lys}^{5\,\mu}\text{(5'}-UCAGACUUUUAACGUA-3')} or E. coli tRNA_{Lys}^{5\,\mu}\text{(5'}-GUUGACGUUUUAAUCAAC-3')} were similarly reactive as ACNase substrates, confirming the assumption that the top three base pairs are not critical for ACNase specificity (9). Arbitrary, all GC stems of the respective ASLs 17 and 18 (5'-GCGGAGCUUUUAACGCGC-3' and 5'-GCGGAGCUUUUAACGCGC-3') abolished the reactivity. A similar effect was exerted by the GC-rich stem of ASL 19, resembling that of E. coli tRNA_{Val}^{5\,\mu}-3' except for a reversed 5' to 3' terminal pair (5'-GCACCUUUUAAAGGCGC-3'). Replacing the G residues of these ASLs with inosine residues (except for the 5'-terminal) restored the reactivity almost to the level of the unmodified reference ASLs in the case of the two arbitrary stem sequences. However, the tRNA_{Val}^{5\,\mu}-3'-like I-rich ASL was an order of magnitude less reactive.

**A PrrC Mutation Renders tRNA_{Lys}^{5\,\mu} More Reactive Than the Natural Substrate**—The D287H, D287N, or D287Q mutations —

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**Discussion**

The tRNA_{Lys}^{5\,\mu} ASL as a Minimal Essential Substrate of ACNase—The reference ASL with the three modified bases of E. coli tRNA_{Lys}^{5\,\mu} was cleaved by ACNase twice as fast as the natural substrate but featured a 3–4-fold reduced affinity to the enzyme, suggesting that the ASL domain contains all or nearly all of the ACNase recognition determinants. The small differences between the ASL and full-sized tRNA in K_cat and K_m are attributed to greater conformational freedom of the ASL. Such flexibility could facilitate attainment of a productive transition-state conformation by the ASL but weaken initial binding to the enzyme. However, the weaker binding of the ASL also leaves open possible interactions between PrrC and other portions of tRNA_{Lys}^{5\,\mu} such as the acceptor region. Namely, mutating the A73 discriminator base inhibits ACNase and truncating the 3'-terminal ACCA sequence relaxes the cleavage site specificity (11). These outcomes could be explained by a weak interaction between PrrC and the acceptor region of tRNA_{Lys}^{5\,\mu}. Accordingly, the A73 discriminator could contribute to substrate specificity, directly or by counter-selecting other tRNAs. We also speculate that truncation of the substrate to contain just the ASL eliminates these interactions that cause misalignment and therefore allows for specific and efficient recognition of the cleavage site.

**Structural ASL Features Favored by ACNase**—Comparing the NMR solution structures of the reference ASL and hypomodified counterparts has suggested that individual base modifications play distinct and partially opposing roles in transforming the disordered, unmodified tRNA_{Lys}^{5\,\mu} ASL into the highly ordered native structure (12). Thus, t6A37 improves stacking with A38, and strengthens the interaction of U33 N3H with the 35p36 phosphate, a hallmark of the anticodon loop U-turn. On the other hand, t6A37 partially destabilizes the ASL stem base pairing, reducing the T_m by about 2 °C. The wobble base side chain mms2U34 also stabilizes the anticodon base stacking and U-turn conformation but partially counteracts the base pair disrupting effect of t6A37. Destabilization of the stem and/or the C32-A38 base pair (21) by t6A37 also opposes the effect of the %39 modification (12, 14, 15). Here we have shown that the ASL base modifications also exert distinct effects on ACNase reactivity (Tables I and II). The underlying causes of these complicated and opposing effects may be understood by considering the contributions of the individual base modifications to the ASL solution structure, as described above. Such a correlation leads us to propose that ACNase favors substrates where the anticodon nucleotides are held in a helical A-RNA conformation and the U-turn conformation is retained but the ASL stem base pairing interactions are modestly destabilized. This assumption is backed by the following arguments. First, t6A37 or mms2A37 that dramatically enhanced the ACNase reaction rate also stabilized the anticodon A-RNA and the loops U-turn conformations and destabilized the anticodon stem and bifurcated C32-A38 base pair (12, 14), suggesting that ACNase favors these structural features. In agreement, %39, which inhibited ACNase activity in certain cases, exerts the opposite effect on ASL base pairing interactions. As for the mild positive effect of mms2U34 on ACNase reactivity, it could reflect a balance between a positive contribution to ACNase reactivity of the enhanced anticodon stacking by this modification and a negative contribution due to the destabilized base pairing. However, part of the ACNase-stimulating effect of mms2U34 may be attributed to a direct interaction with Asp287, the anticodon-recognizing residue of PrrC (an issue to be elaborated below). Abolition of ACNase reactivity by the GC-rich stems, but not the IC-rich, reinforces the notion that base pairing stability of the ASL negatively affects the reactivity. As already mentioned, the higher K_cat and lower K_m of the reference ASL compared with the full-sized tRNA_{Lys}^{5\,\mu} may be attributed to the fact that the upper base pairs of the stem are more constrained within the intact tRNA structure. Hence, the destabilization of the anticodon stem, needed for the productive interaction with ACNase, may be inhibited with intact tRNA_{Lys}^{5\,\mu}. However, as we have seen for the ASLs, the more rigid conformation of the intact tRNA could promote tighter initial binding to the enzyme.
Transplanting the tRNA<sup>Val</sup> UUU anticodon within an otherwise tRNA<sup>Val</sup>-3(UUU) sequence does not elicit detectable ACNase reactivity whereas tRNA<sup>Arg</sup>-UUU is highly reactive (11). The difference between the two chimerical tRNA sequences has been attributed to ill-defined ACNase determinants found outside the anticodon loop and shared by tRNA<sup>Val</sup> and tRNA<sup>Arg</sup> but missing from tRNA<sup>Val</sup>-3. However, in view of the current data, a new interpretation becomes apparent. Namely, the failure to cleave tRNA<sup>Val</sup>-3(UUU) may reflect the greater stability of its GC-rich anticodon stem (4 GC pairs) and the reversed purine-pyrimidine configuration of the two base pairs at the bottom of the stem. The importance of this configuration is suggested by the weaker reactivity of the IC-rich version of the tRNA<sup>Val</sup>-3-ASL, compared with the counterparts at the bottom of the stem. The current comparison uses defined assay conditions of matched ASLs differing only in vitro order has been derived from eclectic data collected otherwise tRNA Val-3(UUU) sequence does not elicit detectable configuration further stabilizes the stem by enhancing G39-A38 base stacking.

A Distinct Wobble Base Side Chain Causes the Reduced Reactivity of tRNA<sup>lys</sup>-3—The relative ACNase reactivities of natural substrate, the completely unmodified version and mutants thereof as well as of the mammalian tRNA<sup>lys</sup>-isoacceptors have suggested the following order of wobble base preference of ACNase: mmm<sup>5</sup>S<sup>4</sup>U<sup>2</sup>U+U>C+mcm<sup>5</sup>S<sup>2</sup>U (11). However, such an order has been derived from eclectic data collected in vitro and in vitro and comparisons of substrates differing in more that just their wobble bases. The current comparison uses defined in vitro assay conditions of matched ASLs differing only in a wobble base modification (Tables I and II). These results confirm the opposing contributions of the bacterial mmm<sup>5</sup>U34 and mammalian mcm<sup>5</sup>U34 side chains to ACNase reactivity, whereas both modifications similarly influence the overall ASL conformation (12, 24). Therefore, the inhibitory effect of mcm<sup>5</sup>U34 on ACNase reactivity suggests a specific interaction between residue Asp<sup>287</sup> of PrrC and the mnm<sup>5</sup>U34 side chain of the natural substrate (11). Since replacing mmm<sup>5</sup>U34 with mcm<sup>5</sup>U34 reduces both <i>K<sub>m</sub></i> and <i>K<sub>cat</sub></i> (Table II), the Asp<sup>287</sup> mmm<sup>5</sup>U34 interaction could influence both substrate binding and the catalytic step.

The detection of PrrC mutations that alter the substrate cleavage specificity and compensate for a missing wobble base modification (11) raised the possibility that some of these mutations would also cause ACNase to prefer mammalian tRNA<sup>lys</sup>-3 over the natural substrate. One interest in such an outcome stems from the role human tRNA<sup>lys</sup>-3 plays as the primer tRNA of reverse transcription in human immunodeficiency viruses (22, 23). Provided that the tRNA anticodon is accessible to ACNase in the primer-template complex, it may be possible to tailor ACNase to discriminate between the free form of tRNA<sup>lys</sup>-3 and the annealed tRNA. Detection of ACNase derivatives more proficient in cleaving tRNA<sup>lys</sup>-3 would constitute a step toward the goal of developing a model system for therapeutics targeted at the primer-template complex. Since the reduced reactivity of tRNA<sup>lys</sup>-3 is likely due to the presence of mcm<sup>5</sup>U34 instead of mmm<sup>5</sup>U34 in the natural substrate, it was expected that tRNA<sup>lys</sup>-3 will be rendered more reactive by some of the PrrC mutations that compensate for the absence of mnm<sup>5</sup>U34 (11). This expectation was confirmed by the in vitro behavior of the D287H allele, which cleaved tRNA<sup>lys</sup>-3 relatively faster than wild type and pseudo-wild type alleles, whereas the latter two alleles were relatively more reactive with <i>E. coli</i> tRNA<sup>lys</sup>-3 (Fig. 4). Mechanistically, the reversal of substrate preference may be explained in that the mmm<sup>5</sup>U34-Asp<sup>287</sup> and mcm<sup>5</sup>U34-D287H pairs form salt-bridges or hydrogen bond interactions of opposite polarities that are vital for substrate binding and/or reactivity. Interestingly, among the PrrC D287 replacement mutants expressed in mammalian cells D287Q showed the highest activity with tRNA<sup>lys</sup>-3, cleaving it to a severalfold higher extent than wild type PrrC.<sup>4</sup> The efficient cleavage of the isolated ASL domain by ACNase also suggests that partially modified tRNA<sup>lys</sup>-3 annealed to the HIV-1 genomic RNA will be recognized by ACNase since the native ASL conformation would be retained in the primer-template complex (27, 28). This expectation has been recently confirmed.<sup>5</sup>

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<sup>5</sup>M. Perlman, C. Isel, R. Marquet, and G. Kaufmann, unpublished results.
Structural Features of tRNA^Lys Favored by Anticodon Nuclease as Inferred from Reactivities of Anticodon Stem and Loop Substrate Analogs
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