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Reverse transcriptase of human immunodeficiency virus can use either human $tRNA_3^{Lys}$ or *Escherichia coli* $tRNA_2^{Gln}$ as a primer in an *in vitro* primer-utilization assay

(retroviruses/DNA replication/drug screening/primer-binding site)

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ABSTRACT **Although the reverse transcriptase (RT) of human immunodeficiency virus (HIV) uses human $tRNA_3^{Lys}$ as a primer of viral genome DNA synthesis *in vivo*, HIV RT binds *Escherichia coli* glutamine tRNA and *in vitro*-made human lysine tRNA with nearly equivalent affinities. We show that HIV RT can use either $tRNA_3^{Lys}$ or $tRNA_2^{Gln}$ as a primer for DNA synthesis *in vitro* without the addition of any other host or viral proteins. *E. coli* $tRNA_2^{Gln}$ can serve as a primer for HIV RT if a primer-binding site sequence complementary to the 3′ end of $tRNA_2^{Gln}$ is at the 3′ end of the template. With this reduced template, the specificity of binding the proper tRNA is due to base-pairing between a bound tRNA to the primer-binding site of the viral RNA template rather than sequence-specific recognition of $tRNA_3^{Lys}$ by RT. If an 8-nucleotide viral sequence 3′ to the primer-binding site is included in the template, then addition of Zn^{2+} or Co^{2+} is required for $tRNA_3^{Lys}$-primed synthesis, and $tRNA_2^{Gln}$ now fails to prime synthesis. The latter result implies that a template sequence adjacent to the primer-binding site and containing 6 nucleotides complementary to the anticodon loop of human $tRNA_3^{Lys}$ plays an active role in tRNA discrimination.**

Replication of the human immunodeficiency virus (HIV) genome is initiated by $tRNA_3^{Lys}$. Eighteen nucleotides (nt) at the 3′ end of the tRNA unfold and base-pair with a specific site in the viral RNA termed the primer-binding site, and the 3′-OH of the tRNA serves to prime template-dependent DNA synthesis (1, 2). This tRNA-primed reverse transcription is carried out by the virally encoded reverse transcriptase (RT). The RT of HIV is a heterodimer of a 66-kDa subunit containing both a polymerase domain and an RNaseH domain and a 51-kDa subunit containing only the polymerase domain (1, 2).

The role played by HIV RT in the specificity of initiation is not entirely established. It has been reported that HIV RT can preferentially bind human $tRNA_3^{Lys}$ to form a binary complex but, that when viral RNA is used as a template, it cannot use enzyme-bound $tRNA_3^{Lys}$ as a primer unless HIV nucleocapsid protein is also present (3). However, experiments with other retroviral systems have indicated that understanding the role of RT in priming is complex. Avian myeloblastosis virus (AMV) RT appears capable of catalyzing the hybridizing of tRNA to the AMV primer-binding site (4). Synthesis using purified AMV RT and added tRNA as a primer has been demonstrated *in vitro* on a viral genome stripped of endogenous primer and other viral proteins (5). For murine leukemia virus, however, it has been suggested that RT alone is not sufficient for synthesis initiation (6).

We have developed an *in vitro* system in which HIV RT can use tRNAs as primers with a synthetic DNA oligonucleotide as a template. Under our assay conditions, the RT of HIV can use $tRNA_3^{Lys}$ as a primer in the absence of any other host or viral protein. RT can use either $tRNA_3^{Lys}$ or *E. coli* $tRNA_2^{Gln}$ as a primer for DNA synthesis if a primer-binding site complementary to the 3′ end of the tRNA is present at the 3′ end of the template oligonucleotide. We observe no significant difference in affinity of HIV RT for *in vitro*-made human $tRNA_3^{Lys}$ or *E. coli* $tRNA_2^{Gln}$, consistent with the hypothesis that any discrimination that occurs upon binding the tRNA *in vivo* must be largely due to modified bases in the tRNA (7). Our results suggest that the specificity of tRNA usage by RT does not result solely from sequence-specific recognition of the tRNA by RT but is also a consequence of base-pairing to a specific primer-binding site and of recognition of viral sequences 3′ to the primer-binding site.

METHODS

Protein Purification. HIV RT was purified from an *E. coli* clone containing the RT gene in an overexpressing vector prepared by D'Aquila and Summers (8). All purification steps were done at 4°C. Cells were lysed by a French press in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.0)/2 mM EDTA/0.02% (wt/vol) 1-hexyl β-D-glucopyranoside (βHG) (Calbiochem)/10% (vol/vol) glycerol. The lysate was centrifuged, and the pellet was extracted with 50 mM Mes, pH 6.0/50 mM KCl/50 mM potassium phosphate/0.02% βHG/10% glycerol (HSE buffer) to yield an extract containing the RT activity. Polyethyleneimine was added to 0.05% (vol/vol) to remove nucleic acid, and the RT activity was precipitated with 60% saturated $(NH_4)_2SO_4$. The ammonium sulfate precipitate was redissolved in HSE buffer and applied to a hydroxyapatite column (Bio-Rad) equilibrated in the same buffer. The column was eluted with a linear gradient from 50 to 250 mM potassium phosphate. Fractions containing RT were pooled, precipitated with $(NH_4)_2SO_4$, redissolved in 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]-propane (Bis-Tris propane), pH 7.0/100 mM $(NH_4)_2SO_4$/0.02% βHG/10% glycerol and applied to a heparin column (Pharmacia) equilibrated in the same buffer. The heparin column was eluted with a linear gradient from 0.1 to 1.0 M $(NH_4)_2SO_4$. Pure protein was stored at 4°C in 50 mM Bis-Tris propane, pH 7.0/100 mM $(NH_4)_2SO_4$/0.02% (wt/vol) βHG/10% glycerol/0.02% (wt/vol) sodium azide. Activity is stable under these conditions for at least 1 yr. Yield was ≈100 mg from 200 g (wet weight) of cells.

When purified RT was passed over a Sephadex G200 column, it eluted as a dimer. SDS/PAGE showed that the purified protein was an equimolar mixture of peptides with molecular masses of ≈66 and 51 kDa. The concentration of

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Abbreviations: RT, reverse transcriptase; HIV, human immunodeficiency virus; AMV, avian myeloblastosis virus; βHG, 1-hexyl β-D-glucopyranoside; nt, nucleotide.

RT was determined by UV absorption, using an extinction coefficient of 3.54 $mg^{-1} \cdot cm^{-1}$, as determined by amino acid analysis. Concentrations expressed in molar terms are for the heterodimer.

tRNA Preparation and Purification. *E. coli* $tRNA_2^{Gln}$ was overexpressed and purified as reported by Perona *et al.* (9). A clone for human $tRNA_3^{Lys}$ was constructed from synthetic oligonucleotides that contained the tRNA sequence flanked by a T7 RNA polymerase promoter and a restriction site (S. C. Schultz, J. Qin, and T.A.S., unpublished work). tRNA is produced from this clone by run-off transcription with T7 RNA polymerase *in vitro* by using a modification developed by Arnez and coworkers (10) of the method of Sampson and Uhlenbeck (11). The *in vitro*-made human lysine tRNA was purified by HPLC by using a diethylaminoethyl-derivatized column (9). About 1 mg of tRNA was purified from a reaction containing 100 μg of tRNA-encoding gene.

DNA Synthesis. tRNA-primed DNA synthesis was done at 40°C in 50 mM Mes, pH 6.0/50 mM KCl/5 mM $MgCl_2$/0.02% βHG/100 μM $ZnSO_4$/30 μM of each deoxynucleoside triphosphate/0.5 μM oligonucleotide template/0.2 μM tRNA containing [α-^{32}P]dTTP or [α-^{32}P]dATP. Reactions were continued for 30 min, and the products were collected by ethanol precipitation. Reaction products were analyzed on either 7.5% polyacrylamide native or 15% polyacrylamide/8 M urea denaturing gels, as indicated, loading equal amounts of each reaction by volume.

Gel-Band-Shift Assays. The affinity of RT for tRNA was measured by gel-band-shift assay (12) using 7.5% polyacrylamide gels cast and run in a 25 mM Tris·HCl/190 mM glycine/1 mM EDTA buffer, pH 8.0. ^{32}P-labeled tRNA was mixed with RT in 5 mM Tris·HCl, pH 7.5/50 mM NaCl/5% glycerol/0.02% βHG. tRNA concentration was ≤7.5 nM. Samples were incubated for 30 min at room temperature before separating on gels. *E. coli* $tRNA_2^{Gln}$ and human $tRNA_3^{Lys}$ were labeled at their 3′ ends by attaching ^{32}P-labeled cytidine 3′,5′-bisphosphate using RNA ligase from T4 phage (New England Biolabs). Alternatively, human $tRNA_3^{Lys}$ was internally labeled with [α-^{32}P]ATP during *in vitro* synthesis, or tRNAs were labeled at the 5′ end with polynucleotide kinase (New England Biolabs). Internally labeled tRNA was purified by passage over a sizing column.

RESULTS

Binding of $tRNA_3^{Lys}$ and $tRNA_2^{Gln}$ to RT. We have used gel-band-shift assays to establish that *in vitro*-made human $tRNA_3^{Lys}$ and *E. coli* $tRNA_2^{Gln}$ both bind to HIV RT in the absence of primer-binding site (Fig. 1). An approximate estimate of the binding constant (K_d) for the interaction of $tRNA_3^{Lys}$ and RT from five repetitions of this experiment is 106 nM. For $tRNA_2^{Gln}$, repeating this experiment four times gave an average binding constant of ≈55 nM. Our estimations of binding constants from gel-shift assays are only accurate to ±15 nM for glutamine tRNA and to ±60 nM for lysine tRNA or about a factor of 2.6. In light of the accuracy of the determination, the difference in binding between the two tRNAs is small. Complete formation of the binary complex occurs over a narrow range of RT concentration, indicating positive cooperativity in the binding of tRNA to RT.

Complexes of RT with either human $tRNA_3^{Lys}$ or *E. coli* $tRNA_2^{Gln}$ showed equivalent resistance to competition from random DNA, as assessed by gel-shift assay, with significant reduction in binding occurring between 5 and 50 μg of calf thymus DNA (Sigma) per ml. Complexes between RT and either tRNA will form to, at least, a total ionic strength of ≈400 mM. Addition of $MgCl_2$ at 5 mM or $ZnSO_4$ at 100 μM also produced no change in binding for either tRNA. No difference in binding was seen when tRNAs were labeled at either the 3′ or 5′ end or when $tRNA_3^{Lys}$ was intrinsically labeled (data not shown).

tRNAs as Primers of DNA Synthesis *in Vitro*. We have developed an assay that can conveniently assess the ability of RT to use tRNAs as primers for DNA synthesis (Fig. 2*A*). The assay uses synthetic DNA oligonucleotides as template strands. The template oligonucleotides contain a sequence complementary to the first 18 bases at the 3′ end of the tRNA whose ability to act as a primer is to be assessed. This sequence is referred to as the primer-binding site by analogy to the primer-binding site of a viral genome. When RT is mixed with tRNA and an oligonucleotide containing an appropriate primer-binding site, the tRNA is partly unwound and becomes base-paired with the complementary region in the template oligonucleotide. DNA synthesis can then occur by addition of bases complementary to the template strand to the 3′ end of the tRNA.

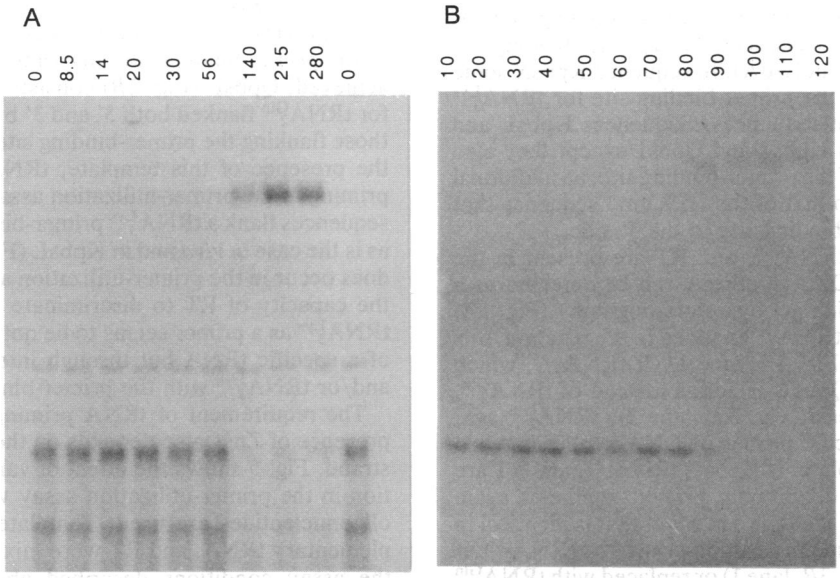

FIG. 1. Autoradiogram showing binding of *in vitro*-synthesized human $tRNA_3^{Lys}$ (*A*) or *E. coli* $tRNA_2^{Gln}$ (*B*) to HIV RT, as revealed by gel-band-shift assay. RT concentration (nM) in the incubation was varied as shown. Other bands in $tRNA_3^{Lys}$ are also products of *in vitro* T7 transcription. The binding constant was estimated to be the point at which half of the tRNA is shifted to the slowly running band.

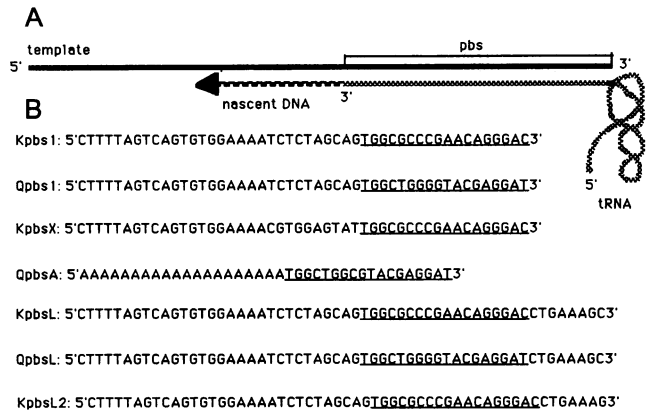

FIG. 2. (*A*) General scheme of assays using tRNA as a primer *in vitro*. A DNA oligonucleotide containing a sequence complementary to 18 bases at the 3′ end of a tRNA (box) is mixed with tRNA and RT. tRNA (represented schematically) becomes partially unwound and base-pairs with the complementary sequence. DNA synthesis is carried out by RT in the direction indicated by the arrow. pbs, primer-binding site. (*B*) Sequences of DNA oligonucleotide templates used; primer-binding sites are underlined. Sequence names that start with K contain a primer-binding site complementary to the 3′ end of human $tRNA_3^{Lys}$; sequence names that start with Q contain a sequence complementary to the 3′ end of *E. coli* $tRNA_2^{Gln}$.

DNA oligonucleotides were used for templates rather than RNA for both convenience and economy. We reasoned that the conformational difference between a DNA–RNA duplex and an RNA–RNA duplex would be small because both would most likely adopt the A form. The short length of these oligonucleotides helps minimize spurious priming, allows easy examination of the length of the reaction products, and, as we learned, is partly responsible for the success of these experiments.

By varying the length and sequence of the flanking regions of the oligonucleotide template, we have observed some intriguing properties of the tRNA-primed synthesis reaction, as we shall describe below. Sequences of template oligonucleotides used in the study are listed in Fig. 2*B*. Sequence Kpbs1 contains 18 nt at its 3′ end complementary to the 3′ end of human $tRNA_3^{Lys}$ and contains 30 nt 5′ to this primer-binding site that are the same as the sequence 5′ to the primer-binding site in the HIV genome. Sequence Qpbs1 is the same, except that it contains a primer-binding site for $tRNA_2^{Gln}$. QpbsA has the sequences 5′ to a primer-binding site for $tRNA_2^{Gln}$ replaced by poly(dA). In sequence KpbsX some Kpbs1 sequences 5′ to the primer-binding site for $tRNA_3^{Lys}$ are replaced by random sequences. Sequences KpbsL and QpbsL are the same as Kpbs1 and Qpbs1 except they also contain, on the 3′ end of the primer-binding site, an additional 8 nt that are identical to that of the HIV viral sequence that flanks the viral primer-binding site to the 3′ side.

If sequence Qpbs1, $tRNA_2^{Gln}$, and RT are present in the assay, the product of DNA synthesis can be detected as a band on a polyacrylamide gel by autoradiography (Fig. 3*A*, lane 2). If, however, no $tRNA_2^{Gln}$ is added to the reaction, this band does not appear (Fig. 3*A*, lane 1). If $tRNA_3^{Lys}$, which cannot base-pair with Qpbs1, is added instead of $tRNA_2^{Gln}$, there is also no synthesis (Fig. 3*A*, lane 3). $tRNA_2^{Gln}$ can, therefore, act as a specific primer of DNA synthesis when Qpbs1 is used as a template. If Kpbs1, $tRNA_3^{Lys}$, and RT are mixed under identical conditions, primed synthesis again occurs (Fig. 3*B*, lane 2), yielding a product that is identical in mobility to that formed in the reaction primed by $tRNA_2^{Gln}$. If $tRNA_3^{Lys}$ is omitted (Fig. 3*B*, lane 1) or replaced with $tRNA_2^{Gln}$ (Fig. 3*B*, lane 3), there is again no synthesis. On the templates Qpbs1 and Kpbs1 $tRNA_2^{Gln}$ and $tRNA_3^{Lys}$ can both act as specific primers.

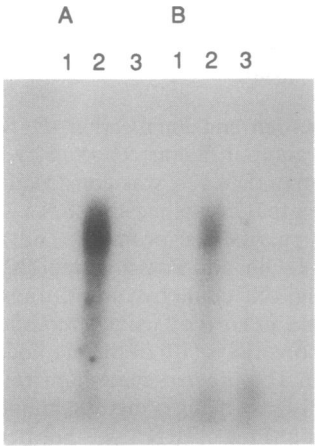

FIG. 3. DNA synthesis by HIV RT with either $tRNA_3^{Lys}$ or $tRNA_2^{Gln}$ used as a primer. Autoradiograph of a native polyacrylamide gel showing products of tRNA-primed DNA synthesis reactions using Qpbs1 (*A*) or Kpbs1 (*B*) as a template separated on a native polyacrylamide gel. tRNA and template oligonucleotide were mixed with RT, as indicated under conditions described in text. Lanes: 1, control reactions without tRNA; 3, control reactions with a tRNA not complementary to the primer-binding site in the template oligonucleotide; 2, products of tRNA-primed synthesis. (*A*) Lanes: 1, Qpbs1 plus RT; 2, Qpbs1 plus RT and $tRNA_2^{Gln}$; 3, Qpbs1 plus RT and $tRNA_3^{Lys}$. (*B*) Lanes: 1, Kpbs1 plus RT; 2, Kpbs1 plus RT and $tRNA_3^{Lys}$; 3, Kpbs1 plus RT and $tRNA_2^{Gln}$.

tRNA-primed DNA synthesis with QpbsA and KpbsX as templates show that sequences 5′ to the primer-binding site are not important for tRNA-primed synthesis (data not shown). If the template utilization assay is done as described above with template QpbsA, which has polydeoxyadenosine 5′ to the $tRNA_2^{Gln}$ primer-binding site instead of viral sequences (Fig. 2*B*), primed synthesis occurs, and controls omitting $tRNA_2^{Gln}$ or replacing it with $tRNA_3^{Lys}$ show no synthesis. Specific synthesis is also seen with KpbsX (which contains some random sequences) and $tRNA_3^{Lys}$. The sequence of the first nucleotides to be copied seems unimportant to priming by $tRNA_2^{Gln}$ or $tRNA_3^{Lys}$. Under conditions where the template strand contains a primer-binding site at its 3′ end, priming by a tRNA molecule requires only that the template strand contain a sequence complementary to the 3′ end of the tRNA.

When viral sequences are added to the template strand 3′ to the primer-binding site, discrimination in primer usage is achieved. QpbsL (Fig. 2*B*) consists of a primer-binding site for $tRNA_2^{Gln}$ flanked both 5′ and 3′ by sequences identical to those flanking the primer-binding site in the HIV genome. In the presence of this template, $tRNA_2^{Gln}$ shows no specific priming in the primer-utilization assay (Fig. 4*A*). When viral sequences flank a $tRNA_3^{Lys}$ primer-binding site both 3′ and 5′, as is the case *in vivo* and in KpbsL (Fig. 2*B*), specific priming does occur in the primer-utilization assay (Fig. 4*B*). Some of the capacity of RT to discriminate among tRNAs and use $tRNA_3^{Lys}$ as a primer seems to lie not in its direct recognition of a specific tRNA but through interactions of the protein and/or $tRNA_3^{Lys}$ with the primer-binding site region.

The requirement of tRNA priming with HIV RT for the presence of Zn^{2+} also depends on the nature of the template strand. Fig. 5 shows the effect of varying $ZnSO_4$ concentration in the primer-utilization assay when different template oligonucleotides are used. Template oligonucleotide, complementary tRNA, and RT were mixed and incubated under the assay conditions described above, except that Zn^{2+} concentration in the assay was varied. Added Zn^{2+} was necessary for the reaction only when an oligonucleotide containing viral sequence 3′ to the primer-binding site was

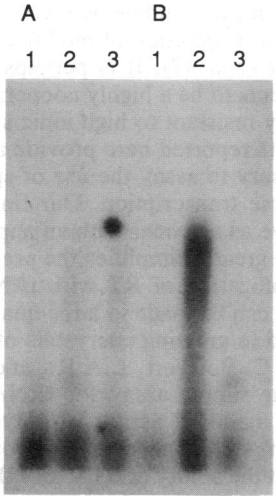

FIG. 4. Viral sequences 3′ to the primer-binding site seem to confer specificity for primer use. Autoradiograph of products separated on a native polyacrylamide gel of tRNA-primed synthesis reactions with QpbsL (*A*) or KpbsL (*B*) as template. Synthesis reaction and controls are in the same order as for Fig. 3. (*A*) Lanes: 1, QpbsL plus RT; 2, QpbsL plus RT and $tRNA_2^{Gln}$; 3, QpbsL plus RT and $tRNA_3^{Lys}$. (*B*) Lanes: 1, KpbsL plus RT; 2, KpbsL plus RT and $tRNA_3^{Lys}$; 3, KpbsL plus RT and $tRNA_2^{Gln}$.

used as a template strand (Fig. 5*D*). When KpbsL2 was the template strand, dependence on Zn^{2+} concentration of $tRNA_3^{Lys}$ being used as a primer is sharp, with no synthesis at 0, 1, or 10 μM $ZnSO_4$ and substantial synthesis at 100 μM and 1 mM $ZnSO_4$. $CoSO_4$ could substitute for $ZnSO_4$ but at $\approx$10 times higher concentration (data not shown).

When the products of synthesis are analyzed under denaturing conditions, the length of the product synthesized using KpbsL2 as a primer substantially differs from that seen for the other template strands (Fig. 5), although the possible number of nucleotides that can be added to each is similar (Fig. 2*B*). The length of the major product in Fig. 5*D*, lane 4

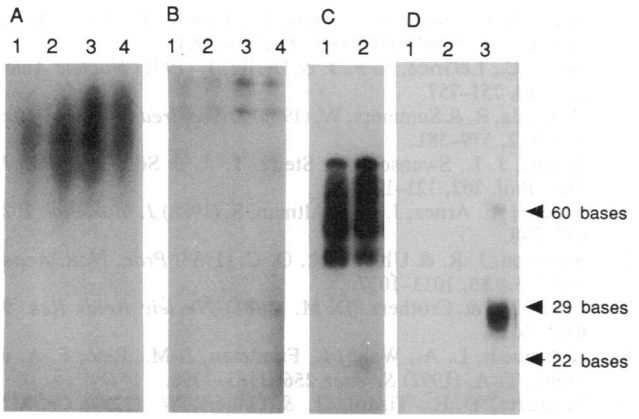

FIG. 5. Effect of Zn^{2+} concentration on ability of RT to use tRNA as a primer in the presence of various DNA oligonucleotide templates. Synthesis reactions were done by mixing RT, tRNA, and template, as described in text, varying $ZnSO_4$ concentration as indicated. Reaction products were separated on denaturing gels and visualized by autoradiography. Mobility of single-stranded ^{32}P-labeled DNA size standards on these gels is indicated. (*A*) RT plus Qpbs1 and $tRNA_2^{Gln}$ incubated with 0, 1, 10, or 100 μM $ZnSO_4$ in lanes 1–4, respectively. (*B*) RT plus KpbsX and $tRNA_3^{Lys}$ incubated with 0, 1, 10, or 100 μM $ZnSO_4$ in lanes 1–4, respectively. (*C*) RT plus QpbsA and $tRNA_2^{Gln}$ incubated without (lane 1) or with (lane 2) 100 μM $ZnSO_4$. (*D*) RT plus KpbsL and $tRNA_3^{Lys}$ incubated with 1, 10, or 100 μM $ZnSO_4$ in lanes 1–3, respectively. Addition of 1 mM $ZnSO_4$ did not increase synthesis beyond the level seen at 100 μM.

appears to be 29 nt, which is the length expected if the tRNA has been cleaved off of the nascent DNA chain (Fig. 2). An appropriate explanation is that the bond between the first base of the nascent DNA strand and the last base of the tRNA primer has been broken but that the primer tRNA remains associated with the template–product complex until denaturing conditions are applied. It is reasonable to suspect that the RNase H activity of RT is responsible for cleaving the RNA–DNA bond between the primer and the nascent DNA.

The band forming a product with an apparent length of $\approx$60 bases in Fig. 5*D*, lane 3 is the result of a self-priming from a hairpin in KpbsL2. When the standard primer-utilization assay is done with KpbsL2 as a template but without $tRNA_3^{Lys}$, a band corresponding to 60 bases is also seen. No synthesis is observed when $tRNA_2^{Gln}$ is substituted for $tRNA_3^{Lys}$ (data not shown). The length of this artifactual product suggests that it represents the addition of a few bases to a partly mismatched hairpin formed by KpbsL2. It is interesting that non-tRNA-primed synthesis, as represented by the self-priming artifact in Fig. 5*D*, lane 3, seems also to depend on added Zn^{2+}. Our purified HIV RT can also be stimulated $\approx$6-fold on oligo(dT)·poly(rA) by Zn^{2+} addition but does not require Zn^{2+} for activity on that template (unpublished data).

We separately varied the amount of tRNA and of template added to a primer-utilization assay to determine a very approximate estimate of the kinetic constants for these substrates (data not shown). When accumulation of reaction products was observed by gel electrophoresis as a function of time, synthesis appeared to proceed at a linear rate for >1 hr. The concentration of tRNA or template was varied, and reaction products were observed at a single time point at 30 min. For tRNA, a concentration of 50–100 nM gave half-maximal synthesis for a 30-min incubation. For the template strand 100 nM was necessary for half-maximal synthesis during this time period. This apparent K_m for tRNA agrees with the binding constant we determined for the association of tRNA with RT, as described above.

DISCUSSION

The ability of HIV RT to use a glutamine tRNA as a primer for template-directed DNA synthesis under certain conditions *in vitro* has implications for the specificity of tRNA binding and priming *in vivo*. Because this enzyme appears to show no preference for binding *in vitro*-made human $tRNA_3^{Lys}$ over *E. coli* $tRNA_2^{Gln}$, RT apparently recognizes features of the tRNA structure common to all tRNAs rather than any specific base sequence; the modified bases of *in vivo*-made $tRNA_3^{Lys}$, however, appear to be important in discrimination leading to the binary complex (7). The specificity of initiation of DNA synthesis by RT with a tRNA primer must lie, in large part, in base complementarity between the unwound end of the tRNA and the primer-binding site.

Our observation that $tRNA_2^{Gln}$ no longer functions as a primer when an 8-nt viral sequence is added to the primer-binding site on the 3′ side is consistent with the possibility that either the enzyme or the tRNA is recognizing template sequences in a manner that affects tRNA binding. The crystal structure of HIV RT and a model of an A-form primer–template complex (13) suggest that interaction is possible between the enzyme and a template strand at $\approx$20–24 nt from the primer terminus. Although we cannot rule out the possibility that (*i*) the additional sequence allows the $tRNA_2^{Gln}$-specific template to adopt an inactive conformation or, conversely, (*ii*) the additional sequence in the $tRNA_3^{Lys}$-specific template allows formation of a secondary structure specifically recognized by RT, these possibilities seem less likely than other alternatives. Most striking is the possibility

of an additional base-pairing interaction between the anticodon of $tRNA_3^{Lys}$ and the template outside the primer-binding site; it is particularly intriguing that 6 base pairs could be formed between 5′ $C_{32}TTTTA_{37}$ of $tRNA_3^{Lys}$ and 6 of the 8 nt (5′-$T_{20}GAAAG_{25}$) 3′ to the primer-binding site, the presence of which causes selective use of $tRNA_3^{Lys}$. The nucleotide sequence of Rous sarcoma virus also contains a site complementary to the anticondon loop of its tRNA primer, although at a distance of 13 nt rather than 3 nt from the 3′ end of the primer-binding site (14).

Zn^{2+} or a related metal ion appears to be important in tRNA-primed synthesis. Although added Zn^{2+} enhances the rate of DNA synthesis on a poly(rA)·oligo(dT) substrate, its addition is not required for this reaction or for tRNA-primed synthesis on some substrates; however, Zn^{2+} is required for DNA synthesis with $tRNA_3^{Lys}$ primer and a DNA template containing a sequence corresponding to the viral primer-binding site and the viral sequences both 3′ and 5′ to the primer-binding site. The Zn^{2+} requirement described here is not due to the presence of tight binding sites like those described for traditional metalloenzymes (15), which are not seen in the crystal structure of RT (13) but rather to a more loosely bound Zn^{2+} used in a different mechanism. For example, Zn^{2+} may have a direct effect on the conformation or secondary structure of the template.

In contrast to Barat *et al.* (3), who concluded that HIV nucleocapsid protein was necessary for tRNA-primed DNA synthesis from full-length viral RNA by HIV RT, we found that this protein was not necessary in our tRNA-primed reactions with very short DNA templates. Thus, nucleocapsid is apparently not absolutely required for tRNA-primed synthesis. Nucleocapsid may, however, further enhance the rate of this reaction or be necessary when the whole genome is present, a possibility not addressed by our studies. *In vivo*, nucleocapsid may bind to the viral genome in a way that affects its secondary structure in a fashion favorable to transcription, such as the melting of hairpins. It will be interesting to see whether a minimum fragment of the viral genome can be defined for which nucleocapsid protein is necessary for tRNA-primed synthesis.

When HIV RT adds DNA nucleotides to a linear RNA primer, the resulting chimeric strand is cleaved at the RNA–DNA junction by the RNase H activity (16). Under certain conditions we also see excision of the tRNA primer from the products of the tRNA-primed reaction. The cleavage of the tRNA from the nascent DNA strand does not occur during the first step but occurs later in the synthesis of the viral genome (1, 2). Cleavage under our assay conditions may be explained by the fact that the template strand in the *in vitro* system is DNA rather than RNA. The RNase H activity of RT can cleave at the junction between DNA and RNA in removing the tRNA primer in AMV (17). In our assay we observe efficient removal of the tRNA only when a short viral sequence adjoins the primer-binding site on the 3′ side. Although our choice of a DNA template strand may affect the step at which the tRNA primer is removed from the nascent DNA strand, this choice is not likely to change the initiation reaction substantially because both double-stranded RNA and DNA–RNA hybrids are probably in a conformation similar to the A form.

Our determination of binding constants for $tRNA_2^{Gln}$ and *in vitro*-made $tRNA_3^{Lys}$ is consistent with the possibility that any specificity of HIV RT for binding $tRNA_3^{Lys}$ to form the binary complex resides in recognition of modified bases, as shown recently by Barat *et al.* (7). It is perhaps unexpected that tRNA binding seems to be a highly cooperative process and that it is relatively resistant to high ionic strength.

The experiments reported here provide a minimum set of conditions necessary to assay the use of specific tRNAs as primers for reverse transcription. Our finding that *E. coli* $tRNA_2^{Gln}$ can serve as a primer with an appropriate complementary template greatly simplifies the preparation of materials for cocrystallization of RT with tRNA and template because $tRNA_2^{Gln}$ can be made in large quantities. We have, in fact, succeeded in growing cocrystals of HIV RT and *E. coli* $tRNA_2^{Gln}$ (D. C. Boisvert, L.A.K., and T.A.S., unpublished work). Our simple assay for tRNA primer use, in conjunction with standard assays of polymerase function, can be used as an additional screen for potential drugs that specifically inhibit the ability of RT to use tRNA as a primer.

Note Added in Proof. Our recent experiments provide evidence for base pairing between the anticodon loop and the template. If the 6-nt sequence that is 3′ to the primer binding site and complementary to the $tRNA_3^{Lys}$ anticodon loop is changed to a sequence complementary to the anticodon loop of $tRNA_2^{Gln}$, priming of transcription by $tRNA_2^{Gln}$ is restored.

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1. Weiss, R. A., Teich, N. M., Varmus, H. E. & Coffin, J. M. eds. (1982) *RNA Tumor Viruses* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
2. Goff, S. P. (1990) *J. Acquired Immune Defic. Syndr.* **3**, 817–831.
3. Barat, C., Lullien, V., Schatz, O., Keith, G., Nugeyre, M. T., Grüninger-Leitch, F., Barré-Sinoussi, F., LeGrice, S. F. J. & Darlix, J. L. (1989) *EMBO J.* **8**, 3279–3285.
4. Araya, A., Sarih, L. & Litvak, S. (1979) *Nucleic Acids Res.* **6**, 3831–3843.
5. Sarih, L., Araya, A. & Litvak, S. (1988) *FEBS Lett.* **230**, 61–66.
6. Prats, A. C., Sarih, L., Gabus, C., Litvak, S., Keith, G. & Darlix, J. L. (1988) *EMBO J.* **7**, 1777–1783.
7. Barat, C., LeGrice, S. F. J. & Darlix, J. (1991) *Nucleic Acids Res.* **19**, 751–757.
8. D'Aquila, R. & Summers, W. (1989) *J. Acquired Immune Defic. Syndr.* **2**, 579–581.
9. Perona, J. J., Swanson, R., Steitz, T. A. & Söll, D. (1988) *J. Mol. Biol.* **202**, 121–126.
10. Vioque, A., Arnez, J. A. & Altman, S. (1988) *J. Mol. Biol.* **202**, 835–848.
11. Sampson, J. R. & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1033–1037.
12. Fried, M. & Crothers, D. M. (1981) *Nucleic Acids Res.* **9**, 6505–6525.
13. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A. & Steitz, T. A. (1992) *Science* **256**, 1783–1790.
14. Schwartz, D. E., Tizard, R. & Gilbert, W. (1983) *Cell* **32**, 853–869.
15. Vallee, B. L. & Auld, D. S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 220–224.
16. Furfine, E. S. & Reardon, J. E. (1991) *Biochemistry* **30**, 7041–7046.
17. Omer, C. A. & Faras, A. J. (1982) *Cell* **30**, 797–805.