THE ROLE OF transfer RNA (tRNA) is not limited to that found in protein synthesis. In fact, tRNA-like structures play an important role in the replication of plant RNA viruses and in regulating the synthesis of the messenger RNAs (mRNAs) of some aminoacyl-tRNA transferases. Moreover, tRNA participates in several other biochemical reactions, including chlorophyll biosynthesis and retrovirus replication.

The first steps of the retroviral cycle involve the binding of the retrovirus to its target cell through the interaction of a receptor in the cell membrane with its viral envelope glycoprotein. Retrovirus virions have a capsid surrounded by an outer envelope. Inside the capsid is a nucleocapsid composed of multiple copies of a small basic protein encoded by gag, one of the conserved retroviral genes. The nucleocapsid protein (NCp15) is derived from the carboxy-terminal region of the Gag protein, and seems to play an important role in several steps of the retroviral life cycle, including the association of the retroviral RNA genome with the nuclear chromatin. Fusion of the target-cell and virion membranes causes entry of the retroviral nucleoprotein complex into the nucleus as part of a nucleoprotein complex. Reverse transcription of the single-stranded (ss) genomic RNA into a double-stranded (ds) proviral DNA, which can be integrated into the host chromosome, is the most probable step during this process. Reverse transcription is a critical step in the replication of retroviruses, and is encoded by a reverse transcriptase (RT) that is an RNA-dependent DNA polymerase.

The fundamental role played by reverse transcriptase in the replication of retroviruses has stimulated the study of the mechanism of action of this enzyme. Reverse transcriptase of the type 1 human immunodeficiency virus forms a stable complex with its cognate transfer RNA replication primer (tRNA\textsuperscript{Lys\textsubscript{3}}). Here, we outline the role of this enzyme in the initiation of its own replication via the complementary region of the retroviral genome, and in the attempts to use the reverse transcriptase–tRNA complex as a new target for antiviral agents.
represents a large step towards understanding structure-function relationships and possibly designing antiviral agents. The structure of the RT-inhibitor complex reveals that the heterodimeric protein has an unprecedented degree of asymmetry. The p66 polymerase domain has a large cleft analogous to that of the DNA-polymerase-containing (Klenow) fragment of *Escherichia coli* DNA polymerase I, which may correspond to the primer-template complex. The p51 subunit, which lacks the p15 domain, has a different structure and no cleft (Fig. 2a). Very recently, the crystal structure of the ternary complex of HIV-1 RT, a monocular antibody Fab fragment, and a dsDNA primer-template complex has been resolved to 3 Å (Ref. 4). The heterodimeric structure of RT in this complex is very similar to that described by Kohlstaedt et al.: the DNA primer-template bound to the enzyme has regions of A-DNA and B-DNA separated by a significant bend.

All DNA polymerases— and RT is no exception to this rule— need a primer carrying a 3'-hydroxyl to initiate DNA synthesis. For the synthesis of the first proviral noncoding-DNA strand, HIV-1 RT uses a tRNA molecule as the primer (Fig. 2b) whereas for the synthesis of the second proviral coding-DNA strand, it uses a short polypurine tract. The tRNA used as a primer differs according to the type of retrovirus (Table 1). In the case of HIV-1, the primer is tRNA~Lys^3~, as deduced from the sequence of the retroviral genome. When isolated from the virion, the 3'-end of primer tRNA~Lys^3~ is found annealed to an 18-nucleotide region called the primer-binding site (PBS), which is located near the 5'-end of the viral genome. The initiation of reverse transcription in all retroviruses examined to date occurs via the addition of a deoxynucleotide to the 3'-end of the cellular tRNA primer. In the case of HIV-1, the PBS consists of nucleotides 183–200 of the HIV-1 RNA genome. Initiation of the noncoding strand leads to production of a short DNA chain ('strong stop') before the first template transfer occurs, which allows the synthesis of the complete noncoding strand. Before the second template-transfer step, during the synthesis of the coding strand of the proviral DNA, primer tRNA is released from the noncoding strand by a single cleavage at the junction between the RNA and the DNA chains.

Similar results have been obtained with other retroviruses. Recently, it was shown that, upon incubation with the HIV-1 RT p66-p51 heterodimer, the noncoding strand resulting from RNase H cleavage retained the 3'-riboadenosine from the original primer tRNA~Lys^3~. By contrast, the cleavage patterns with avian and murine virus RNase H activities were more heterogeneous.

**Selection of primer tRNA**

The primer tRNA found in retrovirus particles originates from the host-cell tRNA population, from which it is selected during virus assembly. Although the host cRNA may contain more than 100 different tRNAs, only a limited population is found within the virions. In the case of HIV-1, tRNA~Lys^3~, tRNA~Lys^2~, tRNA~His^2~ and tRNA~Asp~ are the major tRNA species found in the viral particles. tRNA~Lys^1~ and tRNA~Lys^2~ differ by only one base in the anticodon stem, whereas the difference between these two tRNAs is much larger (Fig. 3a, b). The PBS is not required for incorporation of primer tRNA into the retrovirus, since there is no difference between the profile of incorporated tRNAs in wild-type HIV-1 and those of mutants lacking the PBS.

Several pieces of evidence suggest that RT is involved in the selection of tRNAs found in the virus particles. A strong argument in favour of this role for RT comes from mutants avian sarcoma and murine leukaemia viruses that lack RT, in which the selection of incorporated tRNAs becomes non-specific. In the avian retrovirus, RT forms a stable and specific complex with its primer tRNA~pp~ (for references see Ref. 6).

Using different experimental approaches, including UV crosslinking, RNase footprinting and gel retardation, a complex between animal tRNA~Lys^3~ and HIV-1 RT has been identified. UV crosslinking analysis has shown that HIV-1 RT binds preferentially to the anticodon region of tRNA~Lys^3~. Footprinting analysis showed that, besides the anticodon, the dihydouridine (dihU) loop of tRNA~Lys~ was strongly protected by RT (Fig. 3c, d).

Structural data and studies of the interaction between HIV-1 RT and its primer tRNA suggest that the heterodimeric enzyme is a very flexible protein. Thus, tRNA induces important conformational changes in RT. These have been indicated by fluorescence quenching (HIV-1 RT has a high number of tryptophanyl residues) or by the accessibility of the enzyme to proteolytic attack. Spectrofluorometric measurements revealed two K values for the interactions of tRNA~Lys~ with dimeric HIV-1 RTs: 23 and 140 nM for the p66–p51 form, and 70 and 300 nM for the p66–p66 form. An enzymatically synthesized tRNA~Lys~ formed a complex of lower affinity with RT. These differences may be due to the absence of modified and hypermodified bases in synthetic tRNA~Lys~. Monomeric forms of
HIV-1 RT are devoid of enzymatic activity. A comparison of binding of p66-p51 and isolated p51 and p66 monomers to primer tRNA revealed the monomers could only form complexes at very high protein : tRNA ratios. Both native p66-p51 and the heterodimer reconstituted from its subunits led to recovery of enzyme activity and tRNA binding.

It has been suggested that the specific recognition of tRNALys by HIV-1 RT is less specific than its avian counterpart. HIV-1 RT can bind tRNAs other than its specific primer, although with lower affinity17,21. However, a highly specific interaction between tRNALys and HIV-1 RT has been described14,22.

An important question that has received little attention concerns the structure of RT during the selection of its specific primer. RT is encoded by the pol gene, which is located downstream of the gag gene in the retroviral genome. RT is synthesized as part of the 160 kDa Gag-Pol polyprotein precursor. During virion maturation, the Gag-Pol precursor is cleaved by the virus-encoded protease to yield the mature polymerase. Questions concerning the structure and biological properties of the RT moiety embedded in the Gag-Pol precursor remain to be answered. The cleavage of the Gag-Pol precursor is performed in the budding viral particles, while the selection and annealing of primer tRNA is done before this, after synthesis of viral protein and RNA. It is currently unknown whether or not the polyprotein is involved in interacting with primer tRNA. The presence or absence of RNA-dependent DNA polymerase activity in the polyprotein precursor depends on the type of retrovirus studied. Protease-deficient strains of murine retrovirus have normal levels of RT activity, whereas the avian leukaemia virus RT requires protease cleavage for enzyme activation23. Similar studies have not been carried out, to our knowledge, on HIV-1. The presence of DNA polymerase activity in the murine Gag-Pol precursor indicates that the structure of this polyprotein must have a significant degree of homology to the p66-p51 heterodimer, thus supporting the in vitro studies of the HIV-1 RT–primer-tRNA complex described above. Although the monomeric nature of murine retrovirus RT has been reported, recent evidence indicates that this polymerase dimerizes when it binds to primer-template complex44.

Formation of the PBS–primer-tRNA duplex

Annealing of primer tRNA to the complementary PBS region of the retroviral genome requires the unwinding of two highly ordered structures – the primer tRNA and the RNA viral template. Only a limited region of primer tRNA is annealed to the PBS. The remaining portion of the tRNA molecule (~75 nucleotides) may keep some structural features of native tRNA, and effect the initiation of the replication step by interacting with protein factors or neighbouring regions of the viral template. This idea is supported by the fact that HIV-1 RT can bind and use E. coli tRNA\textsuperscript{GIII} as primer, provided that a PBS sequence complementary to the 3'-end of the bacterial tRNA\textsuperscript{GIII} is present in the DNA template26. However, when short viral sequences are added to the 3'-end of the PBS, selective use of primer tRNA is observed, since use of tRNA\textsuperscript{GIII} is then abolished. This suggests that, in addition to the RT–primer-tRNA recognition step, interactions of RT or primer tRNA with the PBS region are important in primer discrimination. It will be interesting to confirm the latter results by using a viral RNA template.

Footprinting analysis showed that the acceptor stem of tRNALys was more accessible to the nuclease in the pres-
ence of HIV-1 RT, suggesting a partial unwinding of this region. Moreover, a 5' labelled tRNA was more efficiently digested by RT in the presence of HIV-1 RT (S. Litvak et al., unpublished), as shown before for tRNA and avian RT. The partial unwinding of the acceptor stem of primer tRNA is unlikely to be the result of a helicase-like activity of HIV-1 RT, but rather due to structural changes in the acceptor stem of tRNA, induced by the high-affinity binding of RT to the anticodon and diHU regions of primer tRNA.

Synthesis of cDNA, using purified avian myeloblastosis virus RT, tRNA and the viral genome, occurs in vitro in the absence of other viral proteins. A similar observation has recently been reported in which HIV-1 RT, when assayed in vitro, used tRNAs as primers to synthesize cDNA, in the absence of any other host or viral protein, suggesting that only HIV-1 RT is required for the in vitro annealing of primer tRNA to the PBS region. It has been proposed that the nucleocapsid protein NCp15 is also involved in annealing the primer tRNA to the PBS of HIV-1 RNA. A specific association of HIV-1 NCp15 to primer tRNA has been described. However, recent results show that the same protein can bind several tRNAs. NCp15 might improve the tRNA-PBS interaction, since it stimulates the in vitro formation of a complex between primer tRNA and the PBS, as well as the initiation of cDNA synthesis by HIV-1 RT. Very recently, it has been proposed that NCp15 and RT cooperate to select and package primer tRNA during viral assembly. In vivo, NCp15 might also have a synergistic effect on viral replication by unwinding the highly structured retroviral RNA genome.

Modulation of HIV-1 RT activity by tRNA

The search for inhibitors of HIV-1 RT showed that this enzyme is strongly inhibited by some oligonucleotide derivatives. This could be related to the effect of tRNA on the enzymatic activity of HIV-1 RT found during studies on RT-tRNA complex formation and during the development of an in vitro system for cDNA synthesis using HIV-1 RNA as template and tRNA as primer. We observed that tRNA strongly inhibits the DNA polymerase activity. After digestion of tRNA with RNase T1, 12-nucleotide and 14-nucleotide fragments, corresponding to the anticodon regions of tRNA and tRNA respectively, were isolated. These anticodon fragments inhibited HIV-1 RT activity, confirming the importance of the interactions of this region with the enzyme. Moreover, chemically synthesized oligoribonucleotides corresponding to the anticodon and diHU loops inhibited RT at concentrations at which a random sequence or the T-loop did not affect enzyme activity. The inhibitory effect found with tRNA or with native anticodon fragments was stronger than the inhibition by the corresponding synthetic anticodon loop, supporting the hypothesis that modified bases play a role in enzyme-tRNA interactions.

Besides inhibiting RT, tRNA can modulate HIV-1 RT activity in other ways. When RT was incubated with tRNA, a complex formed that was dependent on factors such as time, temperature and tRNA conformation. Stimulation of both RT activities, DNA polymerase and RNase H, followed complex formation. In this case, tRNA
was not used as a primer for CDNA synthesis, but as an enzyme effector, since poly-riboadenosine–oligo-deoxythymidylate was used as the primer–template duplex. The native structure of tRNA is necessary for the stimulatory activity, whereas the inhibitory effect was also obtained with a heat-denatured tRNA in the absence of Mg²⁺ (L. Tarrago-Litvak, unpublished). While kinetic studies showed that the inhibition of HIV-1 RT by tRNA, and oligonucleotides in general, is competitive on the primer–template site of the enzyme, the mechanism of stimulation remains to be established. At least two hypotheses can be raised to explain the stimulatory effect. The conformational changes induced by complex formation between this flexible enzyme and its primer tRNA could lead to an increase in the catalytic capability of the enzyme. Alternatively, primer tRNA could stabilize the dimeric structure of HIV-1 RT, thus stimulating DNA polymerase and RNase H activities, since dimerization is a prerequisite for optimal enzyme activity and tRNA binding.

The finding that tRNA, tRNA fragments and some oligonucleotide derivatives can inhibit HIV-1 RT activity raises the question as to whether or not some tRNA fragments might be potential therapeutic agents against acquired immune deficiency syndrome (AIDS). Oligonucleotides mimicking the protein-binding sequences (‘decoys’) could be used to interfere with specific steps of gene expression. The protein involved in this process could thus be trapped by the pseudosubstrate oligonucleotide and its function inhibited. A successful target for this kind of inhibitor in the retrovirus cycle has been described, since the function of the protein tat, which recognizes the tat region of HIV-1 RNA, is almost totally abolished by using such decoy oligonucleotides. The multifaceted RT is a very attractive target for this kind of inhibitor. The role played by this enzyme, in the selection and annealing of primer tRNA to the viral genome and in the synthesis of dsDNA, makes the interaction of HIV-1 RT with its primer tRNA, or with a primer–template complex, a potential therapeutic target. The observation that tRNA, or some regions of the primer tRNA, can inhibit HIV-1 RT prompted the synthesis of phosphorothioate (S. Litvak et al., unpublished) and dithioate derivatives of the anticondon region and the CCA terminus of tRNA. These acted as very strong inhibitors of HIV-1 RT. This effect could be related to the inhibitory effect of tRNAs described above. Recently, it was shown that RNA pseudoknot structures, a common feature in the folding of the tertiary structure of tRNAs, are powerful and specific inhibitors of HIV-1 RT. These results suggest that the interaction of HIV-1 RT and its primer tRNA, during the first steps of retrovirus replication, is a very attractive target for arresting retrovirus proliferation. In this regard, chemically modified oligonucleotides related to tRNA regions interacting with RT seem extremely promising candidates for developing new antiviral agents.

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References

The authors hope their colleagues will understand that, owing to TIBS policy of short reference lists, several researchers who have made important contributions in this field could not be cited.


Next month in TIBS

The April TIBS has a phosphoprotein flavour, with a Frontlines article on inhibitors of cyclin-dependent kinases and a Talking Point on directing protein tyrosine phosphatases to their correct intracellular locations. There are reviews on the role of Rab proteins in exocytosis; initiation codons within 5’-leaders of mRNAs as regulators of translation; and protein disulphide isomerases. The molecular chaperones series continues with an article on DnaJ-like proteins, and there is an update on numerical integration of rate equations in Computer Corner.