tRNA<sub>Trp</sub> as Primer for RNA-directed DNA Polymerase: Structural Determinants of Function*

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The specific interactions between the RNA-directed DNA polymerase of avian oncornavirus and the tRNA<sub>Trp</sub> primer required for initiation of viral DNA synthesis in vitro were examined. Two distinct interactions, stable binding of the tRNA<sub>Trp</sub> to the enzyme and initiation of viral DNA synthesis by the enzyme with tRNA<sub>Trp</sub> as primer, were characterized as to the structure of tRNA<sub>Trp</sub> required. Different structural features of the tRNA<sub>Trp</sub> were shown to be necessary for each type of interaction. The entire primary structure and native conformation of tRNA<sub>Trp</sub> are both required for binding to reverse transcriptase. Fragments of tRNA<sub>Trp</sub> and intact tRNA<sub>Trp</sub> in an altered conformation cannot be bound by the enzyme using an assay which detects high affinity binding between reverse transcriptase and native tRNA<sub>Trp</sub>. In contrast, fragments of the tRNA<sub>Trp</sub> molecule can serve as primers for viral DNA synthesis with normal efficiency as compared to intact tRNA<sub>Trp</sub>. The fragments which initiate transcription must contain a minimum specific nucleotide sequence which extends from the 3' terminus of the tRNA<sub>Trp</sub> through 27 residues of the molecule. This portion of the tRNA<sub>Trp</sub> may be a major structural determinant of specificity in initiation.

TRANSCRIPTION OF DNA IN VITRO FROM THE NATIVE GENOME OF AVIAN ONCORNAVIRUS BY THE VIRAL RNA-DIRECTED DNA POLYMERASE—(Received for publication, July 10, 1978, and in revised form, August 21, 1979)

Purification of Primer from ASV-tRNA<sub>Trp</sub> was purified by two-dimensional electrophoresis in slab gels (12 x 17 cm); the first dimension was in 10% polyacrylamide for 3.0 h at 350 V, the second in 20% polyacrylamide for 16 h at 350 V. This procedure has been described in detail (5, 16). tRNA<sub>Trp</sub> labeled with <sup>32</sup>P was located in the slab gel by autoradiography, excised, and eluted by shaking the crushed gel in

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The abbreviation used is: ASV, avian sarcoma virus.
0.3 M NaCl at room temperature for 3 to 4 h. The eluate, containing more than 90% of the RNA, was passed through a glass-fiber filter (Reeve-Angel 93A AH) and the RNA filtrate precipitated with ethanol.

**Partial Hydrolysis of Primer by RNase T1**—Partial hydrolysis of \[^{32}P\]tRNA\(^{\text{TM}}\) by RNase T1 was carried out for 1 h at 37°C in 0.5 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl, pH 7.4, with 100 units/ml of RNase T1; the concentration of RNA was adjusted to 500 µg/ml by addition of yeast RNA. After incubation, the reaction mix was diluted to 0.2 M NaCl with 0.02 M Tris-HCl, pH 7.4, extracted with phenol twice, treated with 0.05% diethylpyrocarbonate (Sigma) for 30 s, and then precipitated with isopropyl alcohol. The treatment with diethylpyrocarbonate was shown to have no effect on the polymerase binding assay. The products of RNase T1 hydrolysis were separated by electrophoresis in a 20% polyacrylamide gel (12 × 17 cm) for 4 h at 400 V. Each discrete fragment was excised and eluted by the same procedure as described for the complete tRNA\(^{\text{TM}}\).

**Cleavage of tRNA\(^{\text{TM}}\) with S1 Nuclease**—ASV primer was treated with purified S1 nuclease according to published procedures. \[^{32}P\]-labeled tRNA\(^{\text{TM}}\) was incubated with S1 for 2 h at room temperature in 0.3 M NaCl, 0.03 M sodium acetate, 3 mM ZnCl\(_2\), pH 4.5, in the presence of yeast carrier RNA (100 µg/ml). The RNA was extracted with phenol twice, then precipitated with ethanol. In order to characterize the products, the resultants 3' and 5' halves of the tRNA were separated by electrophoresis in slab gels of 20% acrylamide. RNA fragments were eluted from gel slices as described above, and their position in the tRNA confirmed by nucleotide sequence analysis.

**Isolation of the RNase-resistant Duplex between tRNA\(^{\text{TM}}\) and the ASV Genome**—[^{32}P]tRNA\(^{\text{TM}}\) was renatured with high molecular weight ASV subunit RNA as previously described (15). The reconstituted \[^{32}P\]-labeled primer-template complex was incubated with 50 units/ml of RNase T1 (Worthington) plus 20 µg/ml of RNase A in 0.5 M NaCl, 0.01 M EDTA, 0.02 M Tris-HCl, pH 7.4, for 1 h at 37°C; the RNA concentration was adjusted to 500 µg/ml with yeast RNA carrier. The digestion mixture was diluted 4-fold with 0.01 M EDTA, 0.02 M Tris-HCl, pH 7.4, extracted twice with phenol, treated with 0.05% diethylpyrocarbonate for 30 s, and then precipitated with ethanol. The ASV RNA was resuspended and filtered through a column (45 × 0.9 cm) of agarose A-0.5m in 0.5 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.01 M EDTA, 0.5% sodium dodecyl sulfate. Fractions of 0.6 ml were collected and counted for Cerenkov radiation in a liquid scintillation counter.

**Enzymatic Fragmentation of tRNA\(^{\text{TM}}\)**—Partial hydrolysis of tRNA\(^{\text{TM}}\) with RNase T1, produced at least 10 distinct fragments that could be separated by electrophoresis in a polyacrylamide gel (Fig. 1a; two fragments obtained in relatively low yield B and D are not visible in the reproduction). Each of the fragments was composed of a unique nucleotide sequence, as determined by two-dimensional “fingerprinting” following exhaustive hydrolysis with RNase T1. We also determined the composition of each oligonucleotide in the fingerprints; these data allowed us to locate the fragments within the established nucleotide sequence of tRNA\(^{\text{TM}}\) (Figs. 1 and 2).

**RESULTS**

**Enzymatic Fragmentation of tRNA\(^{\text{TM}}\)**—Partial hydrolysis of tRNA\(^{\text{TM}}\) with RNase T1, produced at least 10 distinct fragments that could be separated by electrophoresis in a polyacrylamide gel. Each of the fragments was composed of a unique nucleotide sequence, as determined by two-dimensional “fingerprinting” following exhaustive hydrolysis with RNase T1. We also determined the composition of each oligonucleotide in the fingerprints; these data allowed us to locate the fragments within the established nucleotide sequence of tRNA\(^{\text{TM}}\) (Figs. 1 and 2).
| Fragments of primer obtained by cleavage with: |
|---------------------------------------------|
| T<sub>1</sub> RNase                        |
| A (27)                                      |
| B (46)                                      |
| C (39)                                      |
| X (38)                                      |
| D (24)                                      |
| E (31)                                      |
| Y (30)                                      |
| F (27)                                      |
| Z (19)                                      |
| J (12)                                      |

| S<sub>1</sub> nuclease                     |
|-------------------------------------------|
| 3' half (38-40)                           |
| 5' half (34)                              |

Fig. 2. Characterization of fragments obtained from tRNA<sup>3'5</sup> by partial hydrolysis with RNase T<sub>1</sub> and S<sub>1</sub> nuclease. tRNA<sup>3'5</sup> was partially hydrolyzed with RNase T<sub>1</sub> or S<sub>1</sub> nuclease and the products separated by electrophoresis in a 20% polyacrylamide gel as shown in Fig. 1, a and b. The fragments identified by letters were eluted from the gel and completely hydrolyzed with RNase T<sub>1</sub>, after which the oligonucleotides were separated by two-dimensional electrophoresis on paper (19). Individual oligonucleotides were eluted and further characterized by hydrolysis with RNase A, followed by electrophoresis on DE51 paper at pH 3.5 (20, 21). The nomenclature identifying the fragments is identical with that in Fig. 1, a and b. * Chain length of each fragment.
2). As a set, the fragments characterized represented all portions of the tRNA except a sequence of 19 nucleotides located at the 5' end of the molecule (Figs. 1 and 2).

Under suitable conditions, S. nuclease cleaves tRNA molecules only in the anticodon and in the nucleotide sequence C-C-Aou at the 3' terminus (17,18). We cleaved tRNAfrP in this manner, separated the resulting molecular halves by electrophoresis in a polyacrylamide gel (Fig. 1b), and then identified the halves by analysis of nucleotide sequence (Fig. 2).

Fragments of tRNAfrP Do Not Bind to Reverse Transcriptase—Panet et al. (8) detected binding of tRNAfrP to reverse transcriptase by filtration of reaction mixtures through columns of Sephadex G-100. We employed the same procedure in our studies and, in preliminary experiments, duplicated the previous findings. When filtered separately through the column, tRNA and polymerase eluted at different positions; polymerase activity was detectable only in the void volume of the column, well ahead of the retarded tRNA. By contrast, when polymerase and tRNAfrP were mixed prior to filtration, they eluted together in the void volume of the column; in these and all of the following experiments, we used polymerase in 100-fold molar excess of RNA in order to obtain maximum binding of RNA to enzyme.

In accord with previous results (8), we found that the reverse transcriptase of ASV specifically bound tRNAfrP, with the exception of tRNAMet, other isoacceptor species of tRNA found in virions of ASV did not bind (data not illustrated). By contrast, specificity of this high order was not observed with another assay for binding, i.e. a filter binding assay (25). Using a filter binding assay, reverse transcriptase readily binds to a variety of viral and nonviral RNAs. Therefore, we employed a stringent assay in the present studies in order to distinguish the exceptional affinity of reverse transcriptase for the tRNA molecule that interact with reverse transcriptase, we subjected the tRNA to partial hydrolysis by RNase T, and then tested the resulting fragments of RNA for their ability to bind to the polymerase. The hydrolyzed RNA (Fig. 4, Lane 1) contained both residual intact tRNAfrP and a set of fragments similar to that illustrated in Fig. 1. Approximately half of the nuclease-treated tRNA bound to reverse transcriptase and eluted in the void volume of the Sephadex columns (Fig. 3A). Analysis by electrophoresis in a polyacrylamide gel revealed that the bound RNA was composed of intact tRNA (Fig. 4, Lane 2) and tRNAfrP molecules lacking adenosine from the 3'-C-C-Aoh terminus (data not shown). However, no fragments were detected even after prolonged autoradiography of the gel.

In an effort to identify specific portions of the tRNAfrP molecule that interact with reverse transcriptase, we subjected the tRNA to partial hydrolysis by RNase T and then tested the resulting fragments of RNA for their ability to bind to the polymerase. The hydrolyzed RNA (Fig. 4, Lane 1) contained both residual intact tRNAfrP and a set of fragments similar to that illustrated in Fig. 1. Approximately half of the nuclease-treated tRNA bound to reverse transcriptase and eluted in the void volume of the Sephadex columns (Fig. 3A). Analysis by electrophoresis in a polyacrylamide gel revealed that the bound RNA was composed of intact tRNA (Fig. 4, Lane 2) and tRNAfrP molecules lacking adenosine from the 3'-C-C-Aoh terminus (data not shown). However, no fragments were detected even after prolonged autoradiography of the gel.

In order to test fragments for binding in the absence of appreciable amounts of intact tRNA, we recovered the unbound RNA from the column and subjected it to a second cycle of binding. Only a small fraction of the RNA bound to reverse transcriptase (Fig. 3B), and this RNA proved to be a trace amount of intact tRNA that had failed to bind in the initial reaction (Fig. 4, Lane 3; RNA not visible in the reproduction). The unbound RNA was composed entirely of fragments (Fig. 4, Lane 4); even the largest fragment produced by hydrolysis with RNase T, (Fragment A, representing 76% of the tRNA molecule) failed to bind.

From the preceding results, we concluded that binding of tRNAfrP to reverse transcriptase requires most if not all of the primary structure of the tRNA. As a final test of this conclusion, we examined the interaction between polymerase and a mixture of 3' and 5' halves of tRNAfrP prepared by hydrolysis with S. nucleas as described above (Fig. 1b). The RNA was denatured to assure separation of the molecular halves and then mixed with reverse transcriptase under conditions for binding. Approximately 30% of the RNA bound to polymerase (data not illustrated), and this RNA consisted only of unfragmented tRNA molecules (Fig. 4, Lane 5); the unbound RNA was composed of the 3' and 5' halves of the tRNA (Fig. 4, Lane 6). We conclude that a single interruption in the primary structure of tRNAfrP (except for the removal of the 3'-terminal C-C-Aoh (17,18)) can ablate the ability of this RNA to bind to reverse transcriptase.

The Duplex Formed Between a Portion of tRNAfrP and a Nucleotide Sequence in the Genome of ASV Does Not Bind to Reverse Transcriptase—A sequence of at least 16 base pairs binds tRNAfrP to the genome of ASV (9,10). This duplex begins with the penultimate nucleotide at the 3' terminus of the tRNA and is therefore immediately adjacent to the site where RNA synthesis initiates; consequently, we hypothesized that the duplex might bind reverse transcriptase as a prerequisite for initiation of DNA synthesis. We prepared the duplex as described previously (9). [32P]tRNAfrP was annealed to denatured unlabeled genome RNA of ASV, the resulting complex was treated with RNase A and RNase T, at high ionic strength, and the surviving duplex RNA isolated by filtration through a column of A-0.5m agarose. The RNase-resistant RNA recovered from the column was found to be homogeneous when analyzed by electrophoresis in a polyacrylamide gel and had the structure and composition de-
FIG. 4. Identification of RNA bound and not bound by ASV polymerase. 
\(^{32}P\)-labeled tRNA\(^{17w}\) was subjected to partial hydrolysis by either RNase T\(_1\) or S\(_1\) nuclease as described under "Experimental Procedures." A portion of each hydrolysate was reserved for subsequent analysis by electrophoresis; the remainder was used in the assay for binding to reverse transcriptase as illustrated by Fig. 3. RNA was recovered from column fractions as described under "Experimental Procedures" and then analyzed by electrophoresis in a slab gel of 20% polyacrylamide at 400 V for 4 h at 15\(^\circ\)C. Radioactive RNA was located by autoradiography. Lane 1, products of partial hydrolysis of tRNA\(^{17w}\) with RNase T\(_1\). Lane 2, RNA recovered from Pool B in Fig. 3A. Lane 3, RNA recovered from Pool B\('^\) in Fig. 3B. Lane 4, RNA recovered from Pool U\('^\) in Fig. 3B. Lane 5, RNA bound to polymerase following partial hydrolysis by S\(_1\) nuclease. Lane 6, RNA not bound to polymerase following partial hydrolysis by S\(_1\) nuclease.

Not all Conformations of tRNA\(^{17w}\) Can Bind to Reverse Transcriptase—We have found that the tertiary conformation of tRNA\(^{17w}\) is altered when the RNA is bound to the ASV genome by hydrogen-bonded base pairs; annealing of tRNA\(^{17w}\) to the viral genome exposes new sites on the tRNA molecule to cleavage by S\(_1\) nuclease.\(^3\) Since the tRNA functions as primer while base-paired with the viral genome, we tested the ability of tRNA\(^{17w}\) in the base-paired form to bind to reverse transcriptase. A substrate suitable for this test was prepared as follows. The duplex, composed of the base-paired region between primer and viral genome, was isolated from the unlabeled native genome of ASV, using hydrolysis with RNase and filtration through agarose as described above. The unlabeled duplex was then denatured and reassociated in the presence of \([\text{\textsuperscript{32}P}]\)tRNA\(^{17w}\), with the denatured duplex in vast excess of the radioactive primer. All of the tRNA reassociated with the complementary strand from the unlabeled duplex and, therefore, migrated more slowly than usual when analyzed by electrophoresis in a polyacrylamide gel (compare Lanes 1 and 3 of Fig. 5). Primer in the reassociated form was recovered from the gel and tested for binding to reverse transcriptase both before and after denaturation of the RNA (Fig. 6A and B, respectively).

Although the reassociated form of primer was poorly resolved from polymerase activity in the assay (Fig. 6A), binding of RNA to enzyme was not apparent. By contrast, release of primer from the reassociated form by denaturation resulted in extensive binding (Fig. 6B); primer released from the reassociated form by denaturation had the electrophoretic mobility of native tRNA\(^{17w}\) (Fig. 5, Lane 2). These findings suggest that the interaction between reverse transcriptase and native tRNA\(^{17w}\) is not identical with the interaction between the polymerase and the primer on the genome of ASV. In order to test this issue further, we performed the experiments described in the following section.

Fragments of tRNA\(^{17w}\) as Primers for DNA Synthesis—The data presented above indicate that the binding of tRNA\(^{17w}\) to reverse transcriptase requires the native structure of the tRNA. In order to determine whether similar requirements govern the initiation of DNA synthesis, we tested fragments of tRNA\(^{17w}\) as primers for the transcription of DNA from the ASV genome.

Nine of the fragments generated by partial hydrolysis of tRNA\(^{17w}\) with RNase T\(_1\) contain the 3' terminus of the tRNA and part or all of the adjacent nucleotide sequence that base-pairs with the viral genome, Fragments A, B, C, D, E, F, and J (Fig. 1a). In order to confirm the identity of these fragments, we exploited our previous observation that the 3' terminus of tRNA\(^{17w}\) can be "tagged" with radioactive dAMP, the nucleotide that initiates transcription from the native ASV genome (11). "Tagged" primer was cleaved with RNase T\(_1\), and the radiolabeled fragments were separated by electrophoresis in a polyacrylamide gel (Fig. 7, Lane 1). Corresponding 3'-ter-

\(^1\)B. Cordell, H. M. Goodman, and J. M. Bishop, manuscript in preparation.
As shown in Fig. 6, tRNA in an altered conformation does not bind to ASV polymerase. Samples of RNA were tested for binding to ASV DNA polymerase by filtration through a column of Sephadex G-100. A, \[^{32}P\]tRNA in an altered conformation. RNA recovered from Lane 1 of the gel illustrated in Fig. 5 and tested for binding to ASV polymerase show no significant binding. B, \[^{32}P\]tRNA in a native conformation. A fraction of the RNA recovered from Lane 1 of the gel illustrated in Fig. 5 was denatured by boiling in water for 5 min and then tested for binding to ASV polymerase. M, \[^{32}P\]RNA; O---O, polymerase activity.

We evaluated the ability of 3'-terminal fragments to form stable duplexes with the genome of ASV by annealing a mixture of uniformly labeled fragments with denatured viral RNA. Fragments bound to the viral genome after the annealing were isolated by filtration through a column of agarose 15m (15), released from viral RNA by denaturation, and then analyzed by electrophoresis in a polyacrylamide gel (Fig. 7, Lane 2). One of the 3'-terminal fragments (I) co-migrates with a major internal fragment (Z) and was, therefore, detected only by the device of "tagging" the 3-terminal fragments with radioactive dAMP.

We then tested whether any of the 3'-terminal fragments could serve as primers for transcription of DNA from the genome of ASV. We cleaved unlabeled tRNA with RNase T1, annealed the resulting fragments of RNA with the denatured genome of ASV, and used the annealed RNA as template-primer for reverse transcriptase with \[^{32}P\]dATP as the only precursor. Fragments A through F were "tagged" by this procedure (Fig. 7, Lane 3) and, therefore, had served as primers for the polymerase. By contrast, Fragments G through K had no detectable primer activity, although all of these fragments possess the 3' terminus of intact tRNA and can form stable base pairs with viral RNA.

In order to further substantiate these findings, we tested the primer activity of fragments in reactions with polymerase as a limiting reagent. These tests were designed on the basis of data generated by varying the amount of polymerase added to reaction mixtures containing a constant amount of template-primer. The primer activity of fragments was tested as described above, using one saturating concentration and two different limiting concentrations of polymerase, 35% and 55% saturation; in all three instances,

![Image](http://www.jbc.org/Downloadedfromhttp://www.jbc.org/)

**Fig. 6.** tRNA in an altered conformation does not bind to ASV polymerase. Samples of RNA were tested for binding to ASV DNA polymerase by filtration through a column of Sephadex G-100. A, \[^{32}P\]tRNA in an altered conformation. RNA recovered from Lane 1 of the gel illustrated in Fig. 5 and tested for binding to ASV polymerase. B, \[^{32}P\]tRNA in a native conformation. A fraction of the RNA recovered from Lane 1 of the gel illustrated in Fig. 5 was denatured by boiling in water for 5 min and then tested for binding to ASV polymerase. M, \[^{32}P\]RNA; O---O, polymerase activity.

**Fig. 7.** Identification of fragments from tRNA that can initiate DNA synthesis by the DNA polymerase of ASV. Samples of \[^{32}P\]-labeled RNAs were analyzed by electrophoresis in a slab gel of 20% polyacrylamide for 4 h at 400 V and 15°C. Lane 1, identification of fragments that include the 3' terminus of tRNA. The 3' terminus of tRNA was labeled with \[^{32}P\]dAMP, the only deoxynucleotide present in a 0.1-ml reaction (i.e. "tagging"), released from template by denaturation at 37°C for 30 min in 90% (v/v) MeSO, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.4, recovered by precipitation with ethanol, and subjected to partial hydrolysis by RNase T1. Lane 2, total set of fragments produced by partial hydrolysis of tRNA with RNase T1. Hydrolysis was performed with tRNA labeled uniformly with \[^{32}P\]orthophosphate. Lane 3, identification of fragments from tRNA that can initiate DNA synthesis. Unlabeled tRNA was subjected to partial hydrolysis by RNase T1; the resulting fragments were annealed to denatured ASV genome and the mixture of reannealed RNAs used as template-primer in a polymerase reaction for "tagging" with \[^{32}P\]dAMP. The RNAs were extracted from the polymerase reaction mixture, denatured as described above, and recovered by precipitation with ethanol. Lane 4, identification of fragments from tRNA that can form stable base-paired structures with the denatured genome of ASV. tRNA labeled uniformly with \[^{32}P\]orthophosphate was partially hydrolyzed with RNase T1. The mixture of \[^{32}P\]RNA fragments was annealed with a 100-fold sequence excess of denatured ASV genome. The bound fragments were purified from unbound fragments by filtration through a column of agarose 15m as described previously (15), denatured and recovered as described above.
the same set of fragments initiated DNA synthesis with roughly comparable efficiencies as judged from the relative intensities of the bands on the autoradiogram (Fig. 8).

The preceding experiments provided only a qualitative test for initiation of DNA synthesis. In order to measure the efficiency of primer activity, we purified Fragments A and C of tRNA<sup>rrp</sup> and then measured the ability of each of these fragments to base-pair with the genome at ASV and to initiate transcription of DNA (Table I). Both of the fragments initiated DNA synthesis with efficiencies approximating the efficiency obtained with complete tRNA<sup>rrp</sup>. We attempted to measure the primer efficiency of several smaller fragments as well, but we were unable to obtain these in sufficient purity to permit decisive measurements.

**DISCUSSION**

We have identified structural features of tRNA<sup>rrp</sup> that are required for two distinct interactions with the reverse transcriptase of ASV, stable binding of the tRNA to the enzyme, and initiation of DNA synthesis by the enzyme with the tRNA as primer. Our data indicate that both the entire primary structure and the native tertiary configuration of the tRNA are probably required for binding to polymerase, whereas a much smaller but specific fraction of the tRNA molecule can serve as primer with normal efficiency.

Our findings with the binding of tRNA<sup>rrp</sup> to reverse transcriptase confirm and extend those reported previously by Haseltine et al. (25). By contrast, Brown and Armentrout (27) have claimed that the 3' half of aminoacylated tRNA<sup>rrp</sup> can bind to reverse transcriptase. Since these authors did not characterize the RNA that bound to polymerase, it is possible that the observed binding was due to uncleaved tRNA.

We have found that annealing of tRNA<sup>rrp</sup> to the genome of ASV exposes new sites on the tRNA molecule to cleavage by S<sub>1</sub> nuclease and must, therefore, alter the tertiary conformation of the tRNA. tRNA<sup>rrp</sup> is bound to the genome of ASV by at least one (9, 10) and perhaps two (28) sets of base pairs that disrupt the acceptor stem and the adjacent loop IV of the cloverleaf and which presumably alter the conformation of the tRNA. In an effort to mimic this conformation we annealed tRNA<sup>rrp</sup> with a complementary sequence of 16 nucleotides derived from the ASV genome; in addition, we isolated the duplex of 16 base pairs formed by this annealing. Neither form of RNA bound to reverse transcriptase. We

### Table I

| Fragment of primer<sup>a</sup> | RNA length (nucleotides) | Input cpm of [32P]tRNA | Fraction annealed to template<sup>a</sup> | [3H]dTMP cpm incorporated | Normalized<sup>b</sup> [3H]-dTMP cpm incorporated | Incorporation relative to that obtained with complete primer<sup>c</sup> % |
|-----------------------------|--------------------------|-----------------------|------------------------------------------|---------------------------|-----------------------------------|-------------------------------------|
| Primer                     | 75                       | (i) 1,756             | 0.74                                     | 8,091                     | 8,091                              | 100                                 |
|                            |                          | (ii) 1,12              |                                         | 12,011                    | 12,011                             | 100                                 |
| A                          | 57                       | (i) 921               | 0.75                                     | 4,712                     | 6,489                              | 85                                  |
|                            |                          | (ii) 32               |                                         | 7,408                     | 11,438                             | 95                                  |
| C                          | 39                       | (i) 1,194             | 0.82                                     | 5,545                     | 4,340                              | 54                                  |
|                            |                          | (ii) 42               |                                         | 8,229                     | 10,175                             | 85                                  |

<sup>a</sup> See Fig. 1a for identification of the fragments.

<sup>b</sup> Two experiments were carried out (i and ii) using the same primer-template complexes.

<sup>c</sup> Results from duplicate samples were averaged after subtraction of the background incorporation obtained by using as template denatured ASV genome devoid of tRNA<sup>rrp</sup>. In experiment i, the background was 750 cpm; in experiment ii, the background was 1510 cpm.

<sup>d</sup> Normalized incorporation = 

\[
\text{(input}^{32P}\text{ cpm primer) (fraction of primer annealed to template)} \\
\text{chain length of primer}
\]

\[
\text{(input}^{32P}\text{ cpm fragment) (fraction of fragment annealed to template)} \\
\text{chain length of fragment}
\]
presume that the duplex structures tested by us failed to bind because they either did not duplicate all of the essential features of the native template-primer or because the binding constants of these structures are lower than the assay can detect.

The affinity between certain tRNAs and the reverse transcriptase of ASV could account for at least two features of virion structure. First, both tRNA^{Tm} and tRNA^{Met} are relatively abundant within virions of ASV (29). Since both tRNAs bind to reverse transcriptase with exceptional affinity (8), and since virions contain roughly equivalent numbers of molecules of polymerase (30) and of the two tRNAs (29), it is possible that binding to reverse transcriptase may facilitate incorporation of these tRNA's into the virion. Second, formation of base pairs between tRNA^{Tm} and the genome of ASV requires disruption of an appreciable portion of the secondary structure of the native tRNA. Perhaps the binding to reverse transcriptase may facilitate incorporation of the native template-primer or because the binding constants of these structures are lower than the assay can detect.

Our data define a sequence of nucleotides within tRNA^{Tm} that is sufficient to serve as primer for transcription from the genome of ASV. The requirement for this sequence appears to be stringent, elimination of two nucleotides from its 5' end reduces or eliminates primer function (compare Fragments F and G, Fig. 7, Lane 3), although appreciably shorter sequences (e.g. Fragments H, I and J) form stable duplexes with the ASV genome and provide the proper 3' terminus for initiation of DNA synthesis. Residues 59 through 75 (see Fig. 9) of the required sequence are nucleotides known to be involved in base-pairing with the ASV genome (9, 10). All but one of the remaining nucleotides in the required sequence (residues 50 to 58, Fig. 9) may also base-pair with viral RNA (28), although this base-pairing has yet to be demonstrated experimentally, it could conceivably account for the role of this portion of the tRNA molecule in the initiation of DNA synthesis.

The primer on the genome of the Moloney strain of murine leukemia virus is tRNA^{Met} (31), which can bind to and initiate DNA synthesis by the reverse transcriptase of ASV (26). The only substantial homology between the nucleotide sequence of tRNA^{Tm} and that of tRNA^{Met} is found in residues 48 to 58, C-G-U-G-ψ-C-G-m'A-Ap for tRNA^{Tm}, C-G-G-G-ψ-C-G-m'A-Ap for tRNA^{Met}. This similarity corroborates our conclusion that these nucleotides are required for the initiation of DNA synthesis.

Both tRNA^{Tm} and tRNA^{Met} have 2 adjacent residues of pseudouridine in loop IV of the cloverleaf replacing the characteristic doublet of ribothymidine and pseudouridine common to most tRNAs (3, 31, 32). Consequently, it has been proposed that these nucleotides are involved in the interaction between the tRNAs and reverse transcriptase (26). Our data neither refute nor confirm this possibility, but we can conclude that the presence of the pseudouridines does not suffice for initiation of DNA synthesis by the polymerase; fragment II contains both the 3' terminus of tRNA^{Tm} and the 2 residues of pseudouridine, yet fails to serve as primer for reverse transcriptase (Fig. 7, Lane 3).

Panet et al. (8) have proposed that the stable binding of reverse transcriptase to tRNA^{Tm} mediates the initiation of transcription from the genome of avian oncornaviruses. The data presented here cast some doubt on this proposal since fractions of tRNA^{Tm} that fail to bind to reverse transcriptase can nevertheless serve as primers for site-specific initiation of DNA synthesis by the polymerase.

We conclude that stable binding between reverse transcriptase and primer is not necessarily a prerequisite for site-specific initiation of transcription from retrovirus genomes. Only a portion of the tRNA^{Tm} is required for initiation of viral DNA synthesis by the polymerase, and the nucleotide sequence of this portion may be a major structural determinant in directing the specificity in initiation.

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