Structural features required for the binding of tRNA^{Trp} to avian myeloblastosis virus reverse transcriptase

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ABSTRACT

The basis of the specific binding of tRNA^{Trp} by avian myeloblastosis virus reverse transcriptase was studied by chemical and enzymatic modification of the RNA. Binding does not depend on recognition of the tryptophan anticodon since molecules cleaved in the anticodon are stably bound by the enzyme. Modification of pseudouridine residues in the tRNA destroys binding to reverse transcriptase. These results are consistent with a model in which reverse transcriptase-tRNA^{Trp} interaction occurs not at the anticodon, but at regions in the tRNA which contain or are stabilized by pseudouridine residues.

INTRODUCTION

Retroviruses contain large amounts of tRNA, which are derived from the host cell and packaged during virus budding. One of the genome-associated tRNAs acts as a primer for in vitro DNA synthesis catalyzed by the viral RNA-directed DNA polymerase, also known as reverse transcriptase (1-14). In the Avian Leukosis-Sarcoma Virus (ALSV) system the primer for DNA synthesis is tRNA^{Trp} (2,3,11,14).

Avian myeloblastosis virus (AMV) reverse transcriptase binds to tRNA^{Trp} in vitro (15-18). It has been proposed that this interaction is responsible for presence of a specific subset of host tRNAs in ALSV particles (18-21). Reverse transcriptase binding may be required for hybridization of the primer tRNA to the genomic template RNA (22).

The specificity of the interaction between the enzyme and the tRNA implies that tRNA^{Trp} contains features which distinguish it from other tRNAs. Two possible features are readily apparent from the nucleotide sequence (23), although others undoubtedly exist. First, the sequence of the anticodon is Cm-C-A, consistent with its identity as a tryptophanyl tRNA. Second, the rT normally found at position 53 is a ψ in this tRNA. In this paper, we report that reverse transcriptase binds to tRNA^{Trp} from which the intact anticodon sequence has been excised, but fails to bind to tRNA^{Trp} in which four pseudouridine residues have been chemically modified by cyanoethylation. These

results indicate that the pseudouridine residues in tRNA^{Trp} effect interaction with the enzyme, either directly or through stabilization of a particular tertiary structure.

MATERIALS AND METHODS

[³²P]-labeled tRNAs.

Chick embryo fibroblasts were grown and labeled with $^{32}\text{PO}_4^{3-}$ as previously described (21). Cytoplasmic RNA was fractionated by centrifugation through 5 ml gradients of 5-20% sucrose in TSE (TSE = 0.1 M NaCl, 20 mM Tris HCl pH 7.8, 1 mM EDTA) + 0.5% Sarkosyl. Centrifugation was at 4°C for three hours at 50,000 rpm in an SW 50.1 rotor. Specific tRNAs were prepared by electrophoresis on two-dimensional polyacrylamide gels (24) of gradient purified 4S RNA. Further purification was achieved by electrophoresis through 16% polyacrylamide (19:1 acrylamide:bis-acrylamide) gels containing 7M urea. RNAs were located by autoradiography of the gels and eluted as described (10,25), except that tRNAs used in binding experiments with reverse transcriptase were eluted and precipitated without addition of yeast carrier RNA unless otherwise noted. tRNA preparations were checked for purity by oligonucleotide fingerprinting (26).

Reverse transcriptase.

Avian myeloblastosis virus DNA polymerase was the gift of Dr. J. Beard (Life Sciences Inc., St. Petersburg, Florida) and was not further purified prior to use.

Binding assay.

Binding of tRNA^{Trp} to AMV reverse transcriptase was performed as described by Panet et al. with slight modification (17). 1-10 ng of [³²P] tRNA^{Trp} was incubated with 20-50 units of reverse transcriptase in 0.05-0.1 ml binding buffer (50 mM Tris HCl pH 8.3, 60 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 5% glycerol, 0.1% Nonidet P-40) for ten minutes at room temperature. The sample was analyzed by gel filtration through a column of Sephadex G-100 (35 x 0.5 cm) pre-equilibrated with 0.1 M potassium phosphate buffer pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40. These buffers differed from the buffers used by Panet et al. (17) in that MgCl₂ was replaced with EDTA. The column was developed with equilibration buffer and 10 drop fractions were collected and assayed directly by counting Cerenkov radiation. Reverse transcriptase activity was assayed by incorporation of ³H-dTMP into trichloroacetic acid insoluble material in an assay mixture containing 50 mM Tris HCl pH 8.3, 60 mM NaCl, 20 mM dithiothreitol, 0.05%

Nonidet P-40, 10 $\mu\text{g/ml}$ poly rA, 5 $\mu\text{g/ml}$ oligo dT 12-18, and 2 μM ^3H -dTTP (>5 Ci/mMole). Reverse transcriptase assays were not done in every experiment, after the elution position of the enzyme was determined for a given column.

Treatment with pancreatic ribonuclease A.

10-20 ng of [^{32}P] tRNA^{Trp} plus 20 μg yeast carrier RNA was preincubated with reverse transcriptase in binding buffer for ten minutes at 25°C. 50 ng of pancreatic ribonuclease A was added and the reaction was incubated another ten minutes at 25°C. The reaction was then applied to a Sephadex G-100 column and fractionated as described above. Material which eluted with reverse transcriptase activity was deproteinized by phenol extraction, diluted 10-fold and precipitated with cetyltriethylammonium bromide at 0°C (27) after the addition of 100 μg of yeast carrier RNA.

Cleavage of tRNA^{Trp} by S1 nuclease.

10-20 ng of [^{32}P] tRNA^{Trp} plus 20 μg yeast carrier RNA was treated with S1 nuclease (28) to cleave the tRNA specifically at the anticodon and the 3' end. The S1 digest was diluted 10-fold into binding buffer without prior denaturation and assayed for reverse transcriptase binding without any treatment to remove the nuclease. Bound RNA was isolated as described above, denatured by heating, and fractionated by electrophoresis on a 20% polyacrylamide gel. Each species of bound RNA was eluted from the gel and characterized by fingerprinting.

Cyanoethylation of pseudouridine in tRNA^{Trp}.

[^{32}P] tRNA^{Trp} was incubated at 37°C for three hours in 50 mM Na Borate pH 9, 25% dimethyl sulfoxide (unless otherwise specified) and 1 M acrylonitrile in order to modify pseudouridylic acid residues (29). Control reactions were treated with the nonreactive analog propionitrile in place of acrylonitrile. The reaction was terminated by ethanol precipitation of the RNA. The extent of the reaction was assayed by two dimensional thin layer chromatography of ribonuclease T2 digestion products (30) of RNase T₁ oligonucleotides or the entire tRNA.

RESULTS

In vitro binding of tRNA^{Trp} to reverse transcriptase.

Figure 1 shows the elution profiles of tRNA^{Trp} and reverse transcriptase from a Sephadex G-100 column run in the presence of EDTA. In the absence of added enzyme, tRNA^{Trp} eluted as a single peak in the included volume of the column (Fig. 1a). When tRNA^{Trp} was mixed with reverse transcriptase prior to

Figure 1. Binding of avian tRNA^{Trp} to AMV reverse transcriptase. Purified ^{32}P labeled chicken cell tRNA^{Trp} was incubated (A) alone or (B) in the presence of 30 units of AMV reverse transcriptase (RTase) as described in the text. The samples were then subjected to gel filtration over a Sephadex G-100 column. Each fraction was assayed for ^{32}P RNA (A, B) by counting of Cerenkov radiation. Reverse transcriptase activity (C) was determined using poly rA-oligo dT as the template-primer for incorporation of ^3H -dTTP into acid-insoluble material.

loading, a substantial fraction of the RNA eluted in the excluded volume of the column (Fig. 1b), as did reverse transcriptase activity.

The intact anticodon of tRNA^{Trp} is not required for binding to reverse transcriptase.

In order to determine whether enzyme binding required an intact anticodon in tRNA^{Trp} , we digested the RNA with pancreatic RNase in the presence and absence of reverse transcriptase or with S1 nuclease. Molecules which could still bind to the enzyme were then isolated and the extent of cleavage which had occurred was determined.

We treated tRNA^{Trp} with pancreatic RNase A while the RNA was complexed with reverse transcriptase. The resulting products were passed over a Sephadex G-100 column and bound material was analyzed by fingerprinting. Figure 2 shows autoradiographs of the RNase T_1 fingerprints of untreated tRNA^{Trp} (Fig. 2a) and tRNA^{Trp} which had been treated with pancreatic ribonuclease in the presence of reverse transcriptase (Fig. 2b). Oligonucleotide 10, which is derived from the anticodon loop of tRNA^{Trp} , was absent from the digest of ribonuclease treated tRNA^{Trp} . The latter RNA had several new

Figure 2. Characterization of tRNA^{Trp} fragments bound by AMV reverse transcriptase. RNase T₁ fingerprints shown are for (A) tRNA^{Trp}, (B) tRNA^{Trp} treated with pancreatic ribonuclease A in the presence of AMV reverse transcriptase, and (C) S1-nuclease cleaved tRNA^{Trp}. The RNAs in both B and C were isolated bound to reverse transcriptase after partial nuclease treatment. (D-F) are schematic representations of the RNAs in (A-C). Arrows in (E) and (F) indicate cleavage sites deduced from the fingerprints in (B) and (C), respectively.

oligonucleotides instead, which were derived from oligonucleotide 10. Three of these new oligonucleotides were identified by RNase T₂ digestion as A-Cm-Up, A-Cm-U-Cm-Cp, and A-¹_mG. Thus, in the presence of reverse transcriptase pancreatic RNase A had cleaved tRNA^{Trp} in at least two sites in the anticodon loop (Fig. 2e), on the 5' side of the anticodon at residue 33 and within the anticodon at residue 35.

S1 nuclease preferentially cleaves free tRNAs at the anticodon loop and at the 3' end of the acceptor stem (28). After treatment of tRNA^{Trp} with S1 nuclease, reverse transcriptase was added and the bound RNA was purified by gel filtration. The bound RNA was denatured by heating and electrophoresed

through a 20% polyacrylamide gel. Two species of RNA bound to the enzyme (data not shown): a minor, slower migrating band with the mobility of intact tRNA^{Trp}, and a major, faster migrating band with the mobility expected for half molecules of tRNA. The faster migrating material was eluted from the gel and fingerprinted.

Figure 2c shows the RNase T1 fingerprint of the faster migrating S1-treated RNA which bound to reverse transcriptase. The fingerprint contained oligonucleotides derived from both the 5' and 3' halves of tRNA^{Trp}, indicating that the bound material was a mixture of both halves of the molecule. Once again, oligonucleotide 10 was destroyed by the nuclease treatment and new oligonucleotides appeared in the bound material. Two of these oligonucleotides were identified as p_m¹Gp and p_m¹G-A-ψ-C-A-Gp, both from the 3' side of the anticodon. Since the S1 nuclease-treated tRNA^{Trp} was not denatured until after binding to the enzyme (just prior to electrophoresis), this material represented whole tRNA molecules nicked at the anticodon.

Modification of pseudouridines in tRNA^{Trp} destroys binding to reverse transcriptase.

tRNA^{Trp} contains four pseudouridylic acid (ψ) residues, including two in the anticodon stem and one at position 53 in place of the highly conserved rT residue. To determine whether any of these residues were involved in the recognition of tRNA^{Trp} by reverse transcriptase, we tested the ability of the enzyme to bind to tRNA^{Trp} after chemical modification of the ψ residues. tRNA^{Trp} was treated with either acrylonitrile, which cyanoethylates ψ residues, or a nonreactive analog, propionitrile, under varying denaturing conditions. To monitor the extent of cyanoethylation the treated RNA was digested with RNase T2 and the digestion products were analyzed by 2-dimensional thin layer chromatography (30). Figure 3 shows the chromatograms of RNase T2 digestion products from modified and unmodified tRNA^{Trp}.

Complete cyanoethylation of the ψ residues destroyed the ability of tRNA^{Trp} to bind to reverse transcriptase. Acrylonitrile treated tRNA^{Trp} eluted from the G-100 column at the position expected for unbound tRNA (Fig. 4a), while the tRNA treated with the nonreactive propionitrile eluted with the polymerase activity (Fig. 4b). When a mixture of modified and unmodified tRNA^{Trp} was passed over G-100 in the presence of reverse transcriptase, two peaks of RNA were observed, corresponding to bound and unbound tRNA. Thus, the cyanoethylation did not produce an inhibitory byproduct. Analysis of the bound and unbound fractions revealed that cyanoethyl-ψ was present only in the unbound fraction (data not shown).

Figure 3. Detection of cyanoethylated pseudouridine (ψ) residues in tRNA^{Trp} . ^{32}P tRNA^{Trp} was incubated with (A) 1 M acrylonitrile or (B) 1 M propionitrile as described in the text. After ethanol precipitation the RNAs were digested with ribonuclease T2 and the digestion products were analyzed by two dimensional thin layer chromatography on cellulose plates (30). Arrows indicate the positions of 1-cyanoethyl pseudouridylic acid (A) and pseudo-uridylic acid (B).

Reaction of tRNA^{Trp} with acrylonitrile under less denaturing conditions (12% vs. 25% DMSO) caused the cyanoethylation of only one ψ residue, at position 38, which is at the base of the anticodon stem. Binding of tRNA^{Trp} to the enzyme was not affected by cyanoethylation of this residue (data not shown). We were unable to differentially cyanoethylate the other three ψ residues in tRNA^{Trp} .

Murine tRNA^{Trp} .

AMV reverse transcriptase binds to other tRNA^{Trp} molecules (31) whose sequences are not identical to that of avian tRNA^{Trp} . For example, mouse

Figure 4. Binding of cyanoethylated tRNA^{Trp} to AMV reverse transcriptase. ^{32}P labeled tRNA^{Trp} was treated with (A) 1 M acrylonitrile or (B) 1 M propionitrile as described in the text. Cyanoethylated and control RNAs were assayed for binding to AMV reverse transcriptase as in Figure 1.

Figure 5. Sequence differences between avian tRNA^{Trp} and the minor murine and bovine tRNA^{Trp}. The main cloverleaf is the structure of avian tRNA^{Trp}. Arrows indicate the positions of base changes found in the minor species of murine tRNA^{Trp} (open letters) and bovine tRNA^{Trp} (boxed letters; 32).

cells contain at least two isoacceptors of tRNA^{Trp}, both of which bind to reverse transcriptase (data not shown). From partial sequence analysis of murine tRNA^{Trp} (J. C. Hu, G. G. Peters and J. E. Dahlberg, unpublished observations) the primary sequence of the major species was found to be the same as that of the avian tRNA^{Trp}, while the sequence of the minor species differed from it in several places. These differences are shown in Figure 5, along with differences between the bovine (32) and avian tRNA^{Trp}.

DISCUSSION

The results presented here indicate that the anticodon loop is not directly involved in the interactions between tRNA^{Trp} and reverse transcriptase. Binding survived nuclease cleavage of the anticodon loop as well as chemical modification of a ψ -residue adjacent to it in the anticodon stem. While these data demonstrate that the anticodon is neither bound to the enzyme nor required for the formation of the complex, they do not reveal which sequences do interact with the enzyme.

Although half-molecules of tRNA^{Trp} held together by non-covalent interactions bind to reverse transcriptase, the purified 3' or 5' fragments of tRNA^{Trp} generated by cleavage at either the anticodon or the 7_mG at residue 46 do not (15,33). Cordell et al. have also demonstrated the inability to a

number of other purified fragments of tRNA^{Trp} to bind stably to reverse transcriptase (33).

A report (34) of the binding of a fragment of tRNA^{Trp} extending from the anticodon to the 3' end seems to conflict with the results presented here. However, it is likely that the bound material observed in that study was equivalent to the SI treated material used in the experiments reported here; i.e., tRNA molecules which were nicked at the anticodon. The 5' half of the molecule complexed with the 3' half would not have been detected in those assays because of the labeling method employed.

In contrast, Baroudy and Chirikjian (35) reported binding of reverse transcriptase to a 67 nucleotide fragment of tRNA^{Trp} which extended from the 5' end of the molecule. That fragment contained all four ψ residues and is consistent with our observation that some of the ψ residues are required for binding. Thus, binding requires neither the acceptor stem nor the anticodon loop, but it does involve regions from both halves of the tRNA molecule.

Araya and Litvak (36) used ultraviolet irradiation to crosslink tRNA^{Trp} to reverse transcriptase. They observed crosslinks between the enzyme and at oligonucleotides derived from the anticodon stem and loop, the dihydrouridine loop and the acceptor end. It was not clear, however, whether or not the crosslinked material represented tRNA bound to the enzyme at the same site and under the same conditions as the material bound in our experiments.

Cyanoethylation of all four ψ residues destroyed the ability of tRNA^{Trp} to bind reverse transcriptase. However, selective cyanoethylation of the ψ residue at position 38 in the anticodon stem did not affect binding, indicating that this residue was not required for enzyme recognition. Since the tRNA was denatured prior to treatment with acrylonitrile, cyanoethylation might have prevented the renaturation of the tRNA into a conformation that could be recognized by the enzyme. Therefore, these results do not necessarily indicate that there is a direct interaction between the enzyme and one or more of the ψ residues at positions 26, 53 or 54.

Although the interaction between avian reverse transcriptase and avian tRNA^{Trp} is likely to involve more than the recognition of primary RNA sequence, it is nevertheless informative to compare the sequences of tRNAs which bind to the enzyme. Bovine and murine tRNA^{Trp} both bind strongly to AMV reverse transcriptase (31, our unpublished observations) although their sequences are not identical to that of chicken tRNA^{Trp}. In addition, other tRNAs with no obvious sequence homologies to that of tRNA^{Trp} show specific, though weak, binding to reverse transcriptase in vitro (20). Thus, we feel that

specificity of binding is determined by several features of the tRNA, such as the ψ - ψ loop or the overall three-dimensional structure of the tRNA. However, we note that since our assay is only qualitative we cannot speak to the possibility that the anticodon or dihydrouridine loops contribute to the strength of the binding.

In contrast to the situation with binding of free tRNA to reverse transcriptase, small fragments of tRNA^{Trp} are capable of priming reverse transcriptase-catalyzed DNA synthesis using ALSV genomic RNA as the template (33). The smallest fragment capable of priming extends from the C-G-U-G immediately upstream from the ψ - ψ sequence through to the 3' C-C-A end. The role, if any, of these sequences in recognition by the enzyme remains unresolved.

The presence of a ψ in place of the highly conserved rT at position 53 is an unusual feature found in two primer RNAs, tRNA^{Trp} (23) and tRNA^{Pro} (37). This substitution is not a universal feature of retrovirus primers, however, since the primer for MMTV has been shown to be a tRNA^{Lys} (9) which contains a rTm in place of the first ψ residue in the G- ψ - ψ -C (38).

From the data obtained by us and others we conclude that loop IV of tRNA^{Trp} is important for both specific enzyme recognition and priming activity. It is unclear whether the ψ residue at position 53 has a direct role in these events. It is likely, however, that the secondary and tertiary structures of this region of the tRNA are responsible, at least in part, for these activities and, perhaps, for the modification of U₅₃ to ψ ₅₃.

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