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$tRNA^{Trp}$ (bovine) binding to the reverse transcriptase of avian myeloblastosis virus and function as a heterologous primer

(RNA-dependent DNA nucleotidyltransferase/transcription/RNA·RNA hybridization/isoacceptor tRNA species)

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ABSTRACT **The primary structures for $tRNA^{Trp}$ (bovine) and primer $tRNA^{Trp}$ (avian) show only minor differences in nucleotide sequence. The heterologous $tRNA^{Trp}$ (bovine) appears to have properties similar to the $tRNA^{Trp}$ (avian) in its ability to bind the $\alpha\beta$ form of RNA-dependent DNA nucleotidyltransferase of avian myeloblastosis virus. A stable enzyme–tRNA complex has been isolated by gel filtration. In addition, $tRNA^{Trp}$ (bovine) can hybridize to the avian viral 35S RNA and act as a primer for transcription of the RNA. $tRNA^{Trp}$ (bovine) can be obtained in larger amounts than the avian primer and can be used to study the interactions between the primer and the viral enzyme.**

The reverse transcriptase (RNA-dependent DNA-nucleotidyltransferase) of avian myeloblastosis virus (AMV) is a Zn^{2+} metalloenzyme (1, 2) that contains: (*i*) polymerase activity directed by RNA; (*ii*) polymerase activity directed by DNA; and (*iii*) ribonuclease H activity. The 64,000 dalton α-subunit has been shown to contain all three activities (3, 4). The $\alpha\beta$ form that contains the 94,000 dalton (β) form appears to have more affinity for synthetic template–primer than the α-subunit. Studies with avian temperature-sensitive enzyme mutants show that all these activities are viral coded (5). A unique characteristic of avian viral reverse transcriptases is their utilization of a tRNA primer for transcription of the high-molecular-weight form of the viral RNA, a property not shared by the cellular polymerases studied to date (6). For the AMV system, this tRNA has been identified as a host $tRNA^{Trp}$ (7). Although all polymerases, with the exception of *Escherichia coli* RNA polymerase, require a primer molecule there appears to be no specific requirement for a host tRNA molecule.

It has been shown that AMV 70S RNA complex is composed of identical 35S subunits together with several tRNA (4 S) and low-molecular-weight (7 S–12 S) RNA molecules (8, 9). The RNA complex can be disrupted by treatment with dimethyl sulfoxide or heat (10). Under melting conditions, the t_m for the release of $tRNA^{Trp}$ primer has the highest value (11). The primer molecules appear to be preferentially annealed at the 5′ end of the viral 35S RNA (12). Based on this location, a speculative model has been proposed for the circular synthesis of the DNA provirus (13). More recently, Haseltine *et al.* (14) showed that DNA chain synthesis in Moloney murine leukemia virus and Rous sarcoma virus are initiated with a tRNA primer at a unique site on the 35S genome. Chicken cell $tRNA^{Trp}$ has been shown to restore the template activity of thermally inactivated 35S RNA from Rous sarcoma virus and AMV sources. Nevertheless, Eiden *et al.* (15), by using oligonucleotide fingerprint analysis, showed that the primer tRNA in AMV is distinct from that observed in RSV. This observation was not confirmed by Waters *et al.* (16). This discrepancy has been discussed further in a recent review by Waters and Mullin (17).

The very limited quantities of avian $tRNA^{Trp}$ primer that can be obtained from the high-molecular-weight viral RNA prompted us to investigate heterologous substitution for the primer molecule. In this paper, we report that bovine liver $tRNA^{Trp}$ is an effective substitute for the avian tRNA primer. The bovine tRNA will bind measurably to AMV reverse transcriptase and has the ability to hybridize to 35S RNA and initiate RNA-directed DNA synthesis.

METHODS AND MATERIALS

Reagents. Labeled and unlabeled triphosphates were obtained from Schwarz/Mann (specific activity, 23 Ci/mmol) for [^{3}H]dTTP; [γ-^{32}P]ATP (ammonium salt) from Amersham/Searle; L-[*side chain-1*-^{14}C]tryptophan and L-[*U*-^{3}H]tryptophan from New England Nuclear; alkaline phosphatase (*E. coli*) from Worthington Biochemical; and polynucleotide kinase from Miles Laboratories, Inc. The RNA markers were purchased from Bethesda Research Laboratories. Yeast $tRNA^{Lys}$ and $tRNA_3^{Leu}$ were prepared in our laboratory. Cellulose ion exchangers were purchased from H. Reeve Angel, Inc.

Virus. The AMV was obtained from the plasma of infected chickens and was provided through contract number N01CP33291 of the Virus Cancer Program of the National Cancer Institute. The virus was purified as described (18).

5′-End Group Labeling of $tRNA^{Trp}$ (bovine). Bovine liver $tRNA^{Trp}$ was prepared essentially by a published procedure (19). Most preparations showed a level of activity of 1800 pmol/A_{260} unit when checked by using bovine liver aminoacyl tRNA synthetase preparations in the aminoacylation assay. One A_{260} unit is equated to 50 μg of tRNA.

The procedure that we followed for ^{32}P-labeling is essentially that of Szekely and Sanger (20). The major steps are: (*i*) removal of the phosphate group at the 5′ end of $tRNA^{Trp}$ (bovine) by *E. coli* alkaline phosphatase; (*ii*) phenol/water extraction of the product, and dialysis of the water layer; (*iii*) phosphorylation of the 5′-hydroxyl end group with [γ-^{32}P]ATP and polynucleotide kinase; (*iv*) a second phenol extraction of the product and then passage of the water layer over DEAE-cellulose. The product, [5′-^{32}P]$tRNA^{Trp}$ (bovine) was dialyzed and had a specific activity of 4×10^7 cpm/A_{260} unit.

The incubation mixture (1.0 ml) contained 10 mM Tris·HCl,/pH 8.0/0.02 mM NaCl/0.5 mM $ZnSO_4$/2.0 A_{260} units of $tRNA^{Trp}$/20 units of alkaline phosphatase. The mixture was incubated at 37° for 30 min, and the reaction was then stopped by adding 50 μl of 0.1 M EDTA and extracting with phenol. For the labeling of the 5′, end group, the reaction mixture (2.0 ml) contained 75 mM Tris·HCl/pH 7.5/10 mM $MgCl_2$/5 mM 2-mercaptoethanol/1.80 A_{260} units of $tRNA^{Trp}$ (bovine)/50 μCi

Abbreviation: AMV, avian myeloblastosis virus.

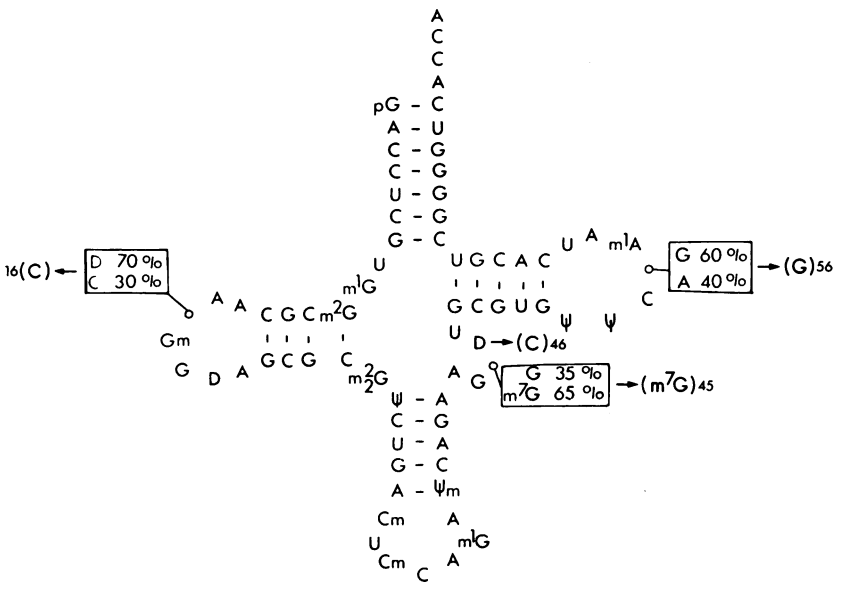

FIG. 1. Primary structure of $tRNA^{Trp}$ from bovine liver. Nucleotides in brackets are those present in $tRNA^{Trp}$ (avian). Boxed nucleotides show the percent distribution of nucleotides at the particular site in $tRNA^{Trp}$ (bovine).

of [γ-^{32}P]ATP/40 units of polynucleotide kinase (specific activity of 30 units/μg). The reaction mixture, after incubating at 37° for 1.5 hr, was extracted with phenol, the water layer was applied to a Sephadex G-25 column to remove free ^{32}P, and the effluent was further purified on a DEAE-cellulose column. The fractions containing primer activity were pooled and dialyzed against 10 mM Tris·HCl, pH 7.5/0.2 mM EDTA.

Preparation of AMV 35S RNA. 35S RNA was extracted from AMV (10), and layered onto linear 5–30% (wt/wt) sucrose gradients in 0.1 M NaCl/0.01 M Tris·HCl, pH 7.3/1 mM EDTA. Samples were centrifuged for 3 hr at 105,000 × *g*. Fractions were collected and the contents precipitated with ethanol.

Enzyme Assay. The AMV DNA polymerase was assayed in a reaction mixture of 0.05 ml containing: 50 mM Tris·HCl, pH 8.3/6 mM $MgCl_2$/1 mM dithiothreitol/50 mM KCl/0.025 mM [^{3}H]TTP or [^{3}H]dGTP (80–300 dpm/pmol)/0.1/A_{260} unit of corresponding template-primer. Reactions were incubated at 37° for 30 min and stopped by transfer to 4°. To each reaction, 0.2 μg of bovine-serum albumin, 0.2 μg of yeast RNA, and 10 μmol of pyrophosphate were added. Cold trichloroacetic acid was then added to achieve a final concentration of 10%. After allowing the reaction mixtures to incubate for 10 min at 4°, acid-insoluble material was collected on Millipore filters. Filters were washed with 10% trichloroacetic acid, and the radioactivity was determined. The product of the reaction was resistant to alkali and RNase but sensitive to DNase. The AMV reverse transcriptase was purified either by conventional methods or by affinity chromatography (21, 22) and subjected to a glycerol gradient centrifugation as a final purification step. A unit of enzyme is defined as the activity that catalyzes the incorporation of 1 nmol of [^{3}H]TTP into acid-insoluble polymer at 37° in 10 min. The specific activity of the purified enzyme used for all experiments was 38,200 units/mg.

Binding Assay. Assays were performed by a modification of a published procedure (23). The reaction mixture contained 50 mM Tris·HCl, pH 7.5/50 mM KCl/10 mM 2-mercaptoethanol/10 mM $MgCl_2$/5% (vol/vol) glycerol/0.1% Nonidet P-40/[^{32}P]$tRNA^{Trp}$ (bovine)/AMV reverse transcriptase as indicated. The reaction mixture was allowed to incubate at 4° for 10 min and then applied to a column (23 × 0.6 cm) of Sephadex G-75 or G-150 previously equilibrated at 4° with buffer containing 100 mM potassium phosphate, pH 7.5/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/10% (vol/vol) glycerol/0.1 mM EDTA/0.1% Nonidet P-40. Fractions (0.19 ml) were collected; 35 μl was used to assay for reverse transcriptase activity and 125 μl was used to detect [^{32}P]$tRNA^{Trp}$ (bovine).

Hybridization of $tRNA^{Trp}$ (bovine) with AMV 35S RNA. All hybridization experiments were performed according to Waters *et al.* (24).

RESULTS

Purification of Bovine Liver $tRNA^{Trp}$. $tRNA^{Trp}$ from bovine liver was purified by classical chromatographic methods. The basic purification scheme involved fractionation of RNA on benzoylated DEAE-cellulose followed by fractionation on DEAE-Sephadex and a final step involving fractionation of the aminoacylated form of $tRNA^{Trp}$ on benzoylated DEAE-cellulose. At this stage, the purified tRNA showed an aminocylation level of 1800 pmol/A_{260} when incubated with the purified homologous activating enzyme. Such preparations were also found to be free of other contaminating tRNA when monitored by the aminoacylation reaction. The ^{32}P-labeled tRNA was also shown to be intact, as judged by the single band obtained on polyacrylamide gel electrophoresis. The primary structure of bovine liver $tRNA^{Trp}$ (19) is shown in Fig. 1. Nucleotides representing differences between the avian and bovine liver $tRNA^{Trp}$ are indicated.

Binding of AMV Reverse Transcriptase ($\alpha\beta$) to[^{32}P] $tRNA^{Trp}$. Complex formation between the $\alpha\beta$ enzyme form and labeled $tRNA^{Trp}$ (bovine) was measured on Sephadex G-75. As shown in Fig. 2*A*, a stable enzyme/tRNA complex was eluted in the excluded volume of the column. To assess the specificity of binding, we used as controls (Fig. 2*B* and *C*) two purified yeast tRNAs ($tRNA_3^{Leu}$ and $tRNA^{Lys}$) whose primary structures differ from that of the host $tRNA^{Trp}$. In both cases, no complex formation could be detected.

Stoichiometry of Enzyme-$tRNA^{Trp}$ Complex. To determine the stoichiometry of interaction, we calibrated a Sephadex G-150 column by using several protein markers (Fig. 3). As can be seen, a major shift occurred in the elution of [^{32}P]$tRNA^{Trp}$ label that indicates the formation of a stable complex, in the

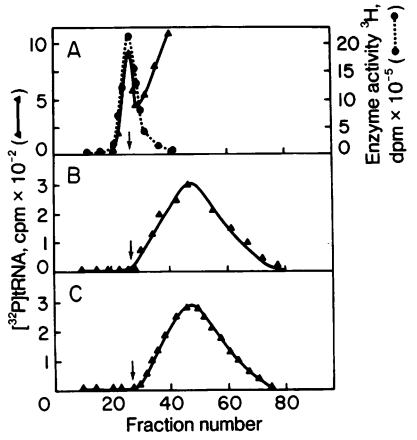

FIG. 2. Complex formation between AMV reverse transcriptase ($\alpha\beta$) (50 units) and tRNA^{Trp} bovine (3.3 × 10^4 cpm): overlap of AMV reverse transcriptase activity and [^{32}P]tRNA^{Trp} (bovine) fractions eluting in the excluded volume of a Sephadex G-75 column and indicating formation of a stable complex under excess tRNA concentrations (no similar complexes were obtained with two yeast tRNAs) (*A*); yeast [^{32}P]tRNA^{Leu} (3.3 × 10^4 cpm) (*B*); and yeast [^{32}P]tRNA^{Lys} (3.0 × 10^4 cpm) (*C*). Arrows indicate elution patterns of polymerase activity.

presence of excess tRNA, with a molecular weight of 190,000 ± 10,000 as determined from the standard curve. Assuming that the complex has a globular structure, we calculate the minimum ratio of the reverse transcriptase to [^{32}P]tRNA^{Trp} (bovine) to be 1:1.

Capacity of tRNA^{Trp} to Hybridize to AMV 35S RNA and Initiate DNA Synthesis *In Vitro*. The AMV 35S RNA devoid of endogenous tRNA primer was prepared as described by Waters *et al.* (24). Hybridization was accomplished by heating the reaction mixture at 80° for 5 min followed by rapid cooling. The sample was then further heated at 65° for 30 min and incubated at 55° for an additional 16 hr. The product was fractionated on a sucrose gradient, and the hybridized complex was separated from free tRNA^{Trp} (Fig. 4). As shown, hybridized [^{32}P]tRNA radioactivity (13,250 cpm) comigrated within the 35S (25 μg) region, and the ratio of bovine liver heterologous RNA primer to viral RNA was 0.83 to 1.0.

In subsequent experiments, the hybridized 35S complex was recovered by centrifugation, and was checked for its ability to initiate DNA synthesis with the four deoxytriphosphates. As shown in Table 1, DNA synthesis occurs in substantial amounts when the heterologous tRNA^{Trp} (bovine) is used, indicating it is an effective substitute.

DISCUSSION

The function of avian host tRNA^{Trp} as a primer for AMV reverse transcriptase is well documented. This tRNA appears to be the only isoacceptor for the amino acid tryptophan, and serves in the dual role of an active primer for AMV reverse transcriptase. Structural studies show identical fingerprint patterns for tRNA^{Trp} (avian) obtained from both host cells and viral core (7, 24, 25). It appears that the entire tRNA molecule is involved in the priming step since the intact (4S) form of tRNA^{Trp} (avian) can be recovered from the AMV 70S RNA complex (7, 25). Studies *in vitro* show that only a fragment (16 nucleotides from the 3'-end) of tRNA^{Trp} (avian) can fulfill this role (26) but the possibility cannot be eliminated that similar fragments may be present in the viral genome. At the present time, there is good evidence that the topology of interaction in the Rous sarcoma virus system between the primer and viral

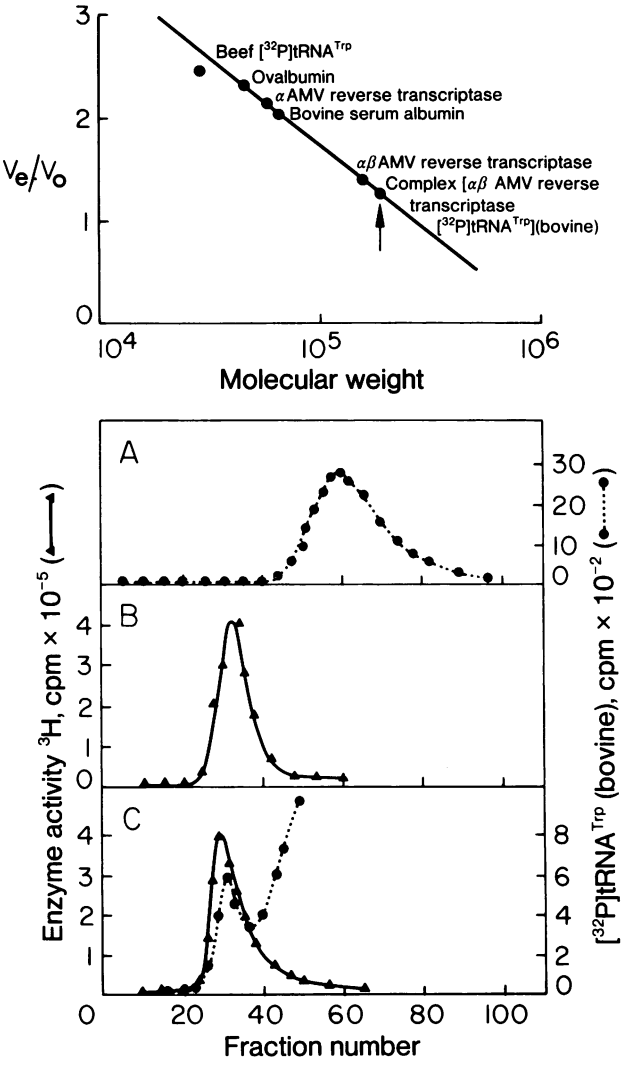

FIG. 3. Determination of the stoichiometry of AMV reverse transcriptase–bovine liver tRNA^{Trp} complex: a Sephadex G-150 column was equilibrated with known molecular weight markers and V_e/V_0 was plotted versus the logarithm of the molecular weights as shown in the top portion of the figure. V_e, elution volume; V_0, void volume. Bottom of figure shows: bovine liver [^{32}P]tRNA^{Trp} (1.6 × 10^5 cpm) (*A*); AMV reverse transcriptase, 50 units of $\alpha\beta$ form (*B*); and isolation of AMV reverse transcriptase ($\alpha\beta$)/[^{32}P]tRNA^{Trp} (bovine) complex from reaction mixture containing 50 units of enzyme and 1.6 × 10^5 cpm of tRNA (*C*).

35S RNA is mainly at the 5'-end of the viral genome. A significant fraction (as much as 30%) of the primer molecules also appears to be bound to pieces of about 20–30 S (12). Interest in this site of interaction has helped generate models for the biosynthesis of circular provirus intermediate (13). Examination of the primary structure of tRNA^{Trp} (avian) and its comparison with tRNAs of known sequences shows the GTψC sequence to be replaced by G$\psi\psi$C in the primer (7, 25, 27). This difference however, is unlikely to play any major role in the secondary and tertiary structure that tRNAs possess because it is also present in several mammalian tRNAs (19).

There are several questions about the unfolding of tRNA^{Trp}, which is a prerequisite to its hybridization to 35S RNA, and its utilization as a primer. It is attractive to speculate that either the polymerase or other proteins, perhaps present in trace amounts, serve to unfold the secondary and tertiary structures (29). Certain tRNAs have been shown to be trapped in a biologically inactive conformation (28). Our studies (unpublished

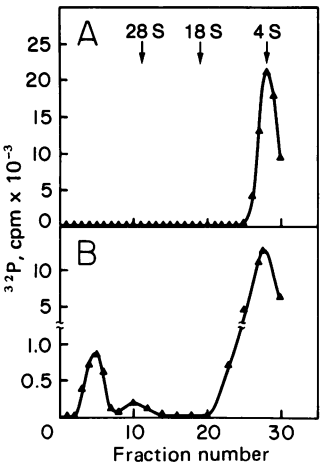

FIG. 4. Hybridization [^{32}P]tRNA^{Trp} to avian 35S RNA: [^{32}P] tRNA^{Trp} (*A*); primer after hybridization to AMV 35S RNA [25 μg of AMV RNA and 13,250 cpm of [^{32}P]tRNA^{Trp} (bovine) were utilized in the hybridization process] (*B*). In addition to free ^{32}P-labeled primer (tRNA^{Trp}), the major peak sedimented in the 35 S region and indicated hybridization between 35S RNA and primer tRNA^{Trp}. ^{32}P-Labeled material in the 28 S region may represent either binding to smaller RNA species (12) or, alternatively, to some nonspecific aggregation product.

results) show that tRNA^{Trp} (bovine) does not appear to be stabilized in the denatured form. Under identical ionic conditions, tRNA^{Trp} can be aminoacylated by its cognate activating enzyme and will bind to AMV reverse transcriptase; this suggests that very similar, if not identical, nucleic acid structures are required by both enzymes.

During the course of our studies dealing with the structure and mode of catalysis by AMV reverse transcriptase, the need arose for substantial amounts of tRNA^{Trp} (avian). Primer can be obtained from virus or host cells in limited amounts; therefore, we systematically searched for a heterologous source of primer tRNA. In this paper, we present data that show tRNA^{Trp} (bovine) to bind effectively to viral 35S RNA, and to serve as a heterologous primer in reverse transcriptions. Comparison of the tRNA^{Trp} (bovine) to the avian primer shows the differences that do not appear to be critical for its biological function. These differences are shown in parentheses (Fig. 1). They include: (*i*) two dihydrouridine residues instead of cytidine residues at positions 16 and 46; (*ii*) an undermethylation of m^{7}G at position 45; and (*iii*) a mixture of 60% guanosine and 40% adenosine at position 56 instead of guanosine solely as found in the avian primer. These differences can be explained by the presence of two isoacceptor molecules in bovine liver or, alternatively, suggest differences in the level of modification. It is of interest to note that, by contrast, tRNA^{Trp} from yeast (30) and *E. coli* (31) show considerable heterogeneity in comparison to the avian primer.

Table 1. DNA synthesis initiated by heterologous tRNA^{Trp} (bovine) using AMV 35S RNA

Sample and incubation time	Acid-insoluble incorporation (dpm)
AMV 35S RNA minus primer*	0
E. coli ribosomal RNA primed with tRNA^{Trp} (bovine)	10,000
AMV 35S RNA primed with [^{32}P]tRNA^{Trp} (bovine)	
45 min	95,054
90 min	214,569
AMV 35S RNA primed with unlabeled tRNA^{Trp} (bovine)	
45 min	87,550
90 min	150,200

All experiments contained the same amounts of primer tRNA^{Trp} (bovine) and AMV 35S RNA. Initiation was carried out after primer was hybridized as described in Fig. 4.
* Endogenous primer removed from 35S RNA.

Our studies show that tRNA^{Trp} (bovine) binds in the aminoacylated (unpublished results) and nonaminoacylated forms to AMV reverse transcriptase in the ratio of 1:1 (if a high affinity constant between the two macromolecules is assumed) (Fig. 3). Moreover, our unpublished results show that no complex between the α-subunit of the enzyme and tRNA^{Trp} (bovine) could be isolated and are in agreement with the work of Grandgenett *et al.* (32). However, this could be a result of the rapid dissociation during gel filtration because of weak binding between the two macromolecules. Stoichiometric values for binding of the tRNA^{Trp} (bovine) to the viral 35S RNA were calculated to be 0.83:1.0 (Fig. 4). This value is within the same range obtained for the avian primer (33). From these results, we conclude that tRNA^{Trp} (bovine) can be effectively substituted for the avian primer based on its primary sequence and biological properties.

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