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Structural requirements for binding of bovine $tRNA^{Trp}$ with avian myeloblastosis virus DNA polymerase

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ABSTRACT

Avian DNA polymerases use host $tRNA^{Trp}$ as the primer for transcription. Bovine $tRNA^{Trp}$ has been previously shown to be a biologic substitute for the avian primer. A bovine $tRNA^{Trp}$ fragment has been identified as having a high binding affinity for the polymerase. The fragment is assigned to be 67 nucleotides, and contains most of the elements required to maintain the secondary and tertiary structure of $tRNA^{Trp}$.

INTRODUCTION

Avian RNA tumor virus DNA polymerases require the host $tRNA^{Trp}$ primer for transcription of the viral genome (1,2). The primer, which is probably hydrogen-bonded to the viral RNA, can only be melted at temperatures higher than that required to remove nonprimer small RNA species from the viral genome (3-5). Chicken-cell $tRNA^{Trp}$ restores the template activity of thermally inactivated 35S Avian myeloblastosis virus RNA, a property not shared by other host tRNAs. The primary sequence of $tRNA^{Trp}$ (bovine) which has been determined is essentially identical with that of the avian $tRNA^{Trp}$ primer (6). Structural studies have shown the 3'-nucleotide tract to be involved in base-pairing to the viral RNA (7-9). AMV DNA polymerase in contrast, was reported to bind to intact $tRNA^{Trp}$ but not to fragments that resulted from nuclease digestion (10, 11).

Our interest has been in studies dealing with the enzymology of AMV DNA polymerase and its interaction with $tRNA^{Trp}$ (6, 12-14). In this paper we report the isolation of a bovine $tRNA^{Trp}$ fragment that binds to AMV DNA polymerase, but does not prime the avian 35S RNA. A preliminary report of this work has appeared previously (15).

METHODS AND MATERIALS

T4 polynucleotide kinase, reagents for polyacrylamide gels, templates for AMV DNA polymerase, and Nonidet-40 were obtained from Bethesda Research Laboratories, Inc. Isotopes were purchased from New England Nuclear. Oligoribonucleotides $A(-A)_2-A(A)_9-$, and poly-rA were purchased from Collaborative Research. AMV DNA polymerase either was obtained from J. Beard and further purified in our laboratory or was purified from AMV virus. The enzyme reaction mixture (0.05 ml) contained: 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 0.025 mM [^{3}H]dTTP or [^{3}H]dGTP (80-300 dpm/pmol), and 0.1 A_{260} unit of a synthetic template-primer.

Preparation of $tRNA^{Trp}$ (bovine): Samples of $tRNA^{Trp}$ were a gift from J. Labouesse (16) and were further fractionated on denaturing polyacrylamide gel elctrophoresis (Fig. 1). Samples were checked for aminoacylation with bovine liver tryptophanyl-tRNA synthetase and the biological activity obtained was 1600 pmol/A_{260}.

[5'-^{32}P]-labelling of $tRNA^{Trp}$ (bovine) with T4 Kinase: $tRNA^{Trp}$ (10 pmol) was dephosphorylated by incubation with 0.1 unit of bacterial alkaline phosphatase in 80 µl of 100 mM Tris-HCl (pH 8.0). After 30 min at 37°, 100 µl (10mM Tris-HCl at a pH of 7.4, 1 mM EDTA) the reaction was stopped by phenol extraction. The dephosphorylated $tRNA^{Trp}$ was recovered by ethanol precipitation and resuspended in 70 µl of 10 mM Tris-HCl (pH 7.4), 1 mM spermidine, and 0.1 M EDTA. To this mixture 10 µl of 500 mM Tris-HCl (pH 9.0), 100 mM $MgCl_2$, 50 mM dithiothreitol, 10 pmol of [^{32}P]ATP and 5 units of T4 polynucleotide kinase was added for a final reaction volume of 100 µl. Incubation at 37° was carried out for 30 min, and tRNA was recovered (17).

Denaturing Polyacrylamide Gel Electrophoresis and tRNA Recovery: Slab gels (0.3 cm x 320 cm^2) were composed of 9.7% acrylamide, 0.3% bisacrylamide, 7 M urea, 50 mM Tris-borate (pH 8.3), and 1 mM EDTA. Pre-electrophoresis was carried out at 10 v/cm for 2 h with 50 mM Tris-borate, pH 8.3, and 1 mM EDTA as the equilibrating buffer. Transfer RNA samples were subjected to electrophoresis at 20 v/cm for 5 h. Transfer RNA and tRNA fragments were extracted from polyacrylamide gels as described elsewhere (17).

Ribonuclease T_1 Partial Digestion of [^{32}P]$tRNA^{Trp}$: Intact [^{32}P]$tRNA^{Trp}$, 1 µg (2.0×10^6 dpm), was incubated with 5 units of T_1 RNase at 47° for 13 min. The incubation mixture contained 20 µg of unlabeled $tRNA^{Trp}$, 25 mM Tris-HCl (pH 6.9), 7 M urea, and 1 mM EDTA. After incubation, fragments were dissolved in electrophoresis buffer and subjected to analysis on polyacrylamide gels.

5'-Nucleotide Analysis of $tRNA^{Trp}$ Fragment: Hydrolysis for 5'-end-group analysis of [^{32}P]$tRNA^{Trp}$ (700 dpm) was carried out in 20 µl of 0.3 N NaOH at 37° for 18 h. The resulting labeled nucleotide diphosphate was identified by electrophoresis on 3 mM paper equilibrated with 0.05 M ammonium formate buffer (pH 3.5), at 20 v/cm.

Enzymatic Degradation of poly rA to Generate Nucleotide Markers: Poly rA was digested by an enzyme isolated from Haemophilus influenzae (Georgetown University). Details of the preparation and properties of the enzyme will be published elsewhere. Under limiting conditions, the enzyme will generate free 5'-hydroxyl oligonucleotides from $A(-A)_2$ to the size of the starting polynucleotide, which can be labeled by T4 polynucleotide kinase.

RESULTS

Binding of a $tRNA^{Trp}$ Fragment to AMV DNA Polymerase: Intactness of $tRNA^{Trp}$ was checked by analysis on a 20% polyacrylamide gel containing 7 M urea. When gels were stained with ethidium bromide, the single band that appeared for $tRNA^{Trp}$ was observed verifying the absence of internal nicks (Fig. 1A), although sequential treatment with alkaline phosphatase and T4 polynucleotide kinase (Fig. 1B) revealed some degradation of $tRNA^{Trp}$. The major band corresponding to intact $tRNA^{Trp}$ was extracted and was shown to be a single species (Fig. 1C) and this sample was used for the remainder of the experiments.

Fragments obtained from partial digestion of $tRNA^{Trp}$ with T_1 RNase were labeled with phosphorus-32 at the 5'-position and then incubated with AMV DNA polymerase to determine complex formation (6, 15). The mixture was fractionated by Sephacryl-S200 chromatography. A small peak that eluted earlier than the $tRNA^{Trp}$ fragments was recovered (Fig. 1E). Fragments from the two peaks were examined by polyacrylamide gel analysis (Fig. 1E), and the lane numbers corresponded to the fractions from the chromatographic step. A partial T_1 RNase digest of $tRNA^{Trp}$ is shown in Fig. 1D.

The labeled $tRNA^{Trp}$-fragment that differed from intact $tRNA^{Trp}$ by the absence of nucleotides from the 3'-end was examined for its capacity to bind to AMV DNA polymerase and to be used as a primer for the transcription of viral 35S RNA. A stable complex of $tRNA^{Trp}$ fragment with AMV DNA polymerase was isolated by Sephacryl-S200 chromatography (Figure 1). Under conditions when intact $tRNA^{Trp}$ was hybridized to the viral genome the fragment was not a substitute (Figure 2) and therefore was not a functicnal primer (Table 1).

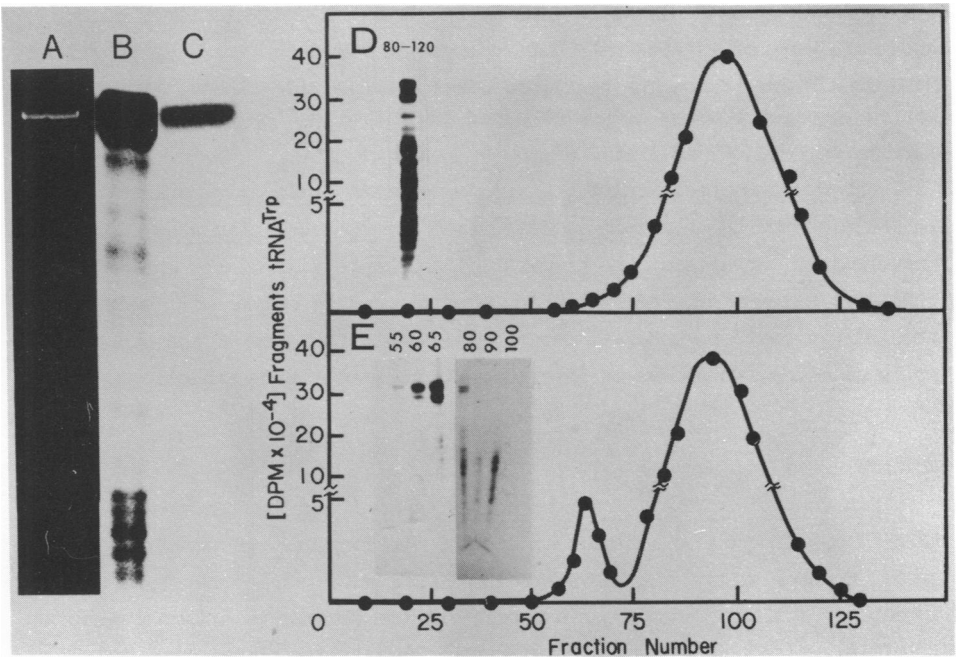

Fig. 1. Analysis of AMV DNA polymerase and [^{32}P]-labeled fragments obtained by T_1 RNase digestion of $tRNA^{Trp}$. Lane A: 10 µg of $tRNA^{Trp}$ before labelling, stained with ethidium bromide. Lane B: $tRNA^{Trp}$ after labeling at the 5' position; minor bands are due to degradation generated by contaminating nucleases. Lane C: intact labeled $tRNA^{Trp}$ after reextraction. Panel D: elution profile of partial T_1 RNase digest of $tRNA^{Trp}$ from a Sephacryl-S 200 column (0.7 x 50 cm), equilibrated with 50 mM phosphate (K^+) (pH 7.0), 50 mM KCl, 2 mM $MgCl_2$, and 0.5 mM EDTA. Panel E: complex of AMV DNA polymerase and tRNA fragment. D and E correspond to fractions from the chromatographic steps.

Sequence assignment for $tRNA^{Trp}$ fragment: The size of the $tRNA^{Trp}$ fragment was measured on a denaturing 20% polyacrylamide gel that was calibrated with the oligonucleotides $A(-A)_5$ to $A(-A)_{65}$ (Fig. 3A). The upper limit of oligonucleotide resolution with the retention of the A_5 to A_9 standards was A_{52} nucleotides (Fig. 3B). When the electrophoretic fractionation was carried out for longer periods, larger oligonucleotides were resolved (Fig. 3F). The size of the $tRNA^{Trp}$ fragment that bound to the polymerase was extrapolated to 67 nucleotides; a small amount of a 64-nucleotide fragment was also noted after longer exposure of the gel to the x-ray film

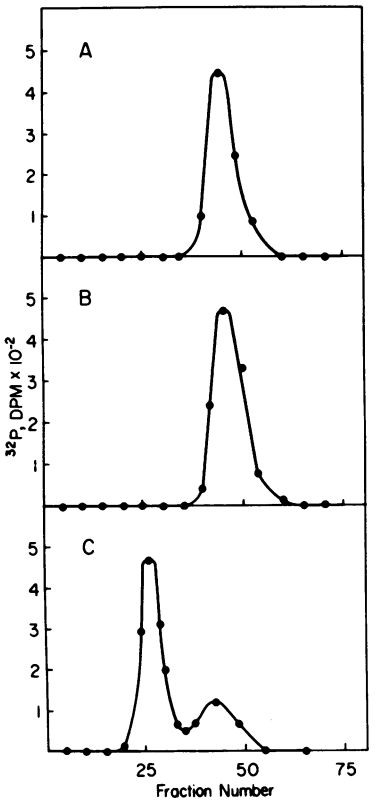

Fig. 2. Binding properties of $tRNA^{Trp}$ fragment to AMV 35S RNA. (A) Displays the elution profile of [^{32}P]-$tRNA^{Trp}$ fragment on Sephacryl-S 200. (B) Shows elution after subjecting the $tRNA^{Trp}$ fragment to hybridiztion conditions to 35S RNA. (C) Control experiment that shows hybridization of intact [^{32}P]- $tRNA^{Trp}$ to 35S RNA. Conditions for hybridization are as described previously (6).

(Fig. 3E). End-group analysis of alkali hydrolyzed labeled $tRNA^{Trp}$ showed the label to have the same mobility as the $_{p}G_{p}$ marker (Fig. 4). These results show the major $tRNA^{Trp}$ fragment to be 67 residues long and to have an intact 5'-end.

DISCUSSION

The mechanism by which AMV DNA polymerase recognizes the $tRNA^{Trp}$ primer remains to be determined. Several experimental approaches have been used to determine the structural requirements for binding of $tRNA^{Trp}$ to the polymerase. This binding and the ability to prime the 35S viral genome have been used as measures of specificity (6,10,11). Such studies were deficient in that they tested only specific fragments and therefore could not support definitive conclusions. Our approach was to identify specific fragments from a partial T_1 RNase digest of $tRNA^{Trp}$ (bovine) that preferentially bind to

TABLE I

TRANSCRIPTION OF AMV 35S RNA INITIATED BY INTACT $tRNA^{Trp}$ (BOVINE) AND $tRNA^{Trp}$ FRAGMENT

Sample*	Acid-insoluble Incorporation (dpm)
AMV 35S RNA (minus primer)	525
AMV 35S RNA primed with [^{32}P] $tRNA^{Trp}$ (bovine)	3,166
AMV 35S RNA primed with [^{32}P] $tRNA^{Trp}$ fragment	184

*Reaction mixture (50 µl) contained 50 mM (pH 8.3), 6 mM $MgCl_2$, 4 mM dithiothrietol, 50 mM KCl, 0.5 mM each of dTTP, dCTP, dATP and 0.2 mM [^{3}H] dGTP 300 dpm/pmol, 35S AMV RNA primed with $tRNA^{Trp}$ or fragment obtained from T_1 RNase digestion of $tRNA^{Trp}$ and AMV DNA polymerase.

the polymerase. The partial digest generated a large variety of overlapping fragments from the $tRNA^{Trp}$ (bovine) which we had previously shown to be a biologic substitute for the avian primer (6).

A fragment obtained from $tRNA^{Trp}$ formed a stable complex with the polymerase which was later assigned as a 67-nucleotide entity with an intact 5'-end. Examination of the primary sequence of $tRNA^{Trp}$ showed sequential G residues at positions G_{66} to G_{69} (Fig. 5). Because the size of the fragment was extrapolated from oligo rA markers which may differ slightly from heteropolymers in migration we cannot exclude other sites between G_{66} and G_{69} as targets for T_1 RNase. We have assigned the fragment as being 67 residues long because G_{67} is part of a non Watson-Crick base pair and is therefore more susceptible to nuclease digesiton (18). We considered identifying the fragment by further digestion, but the four sequential G residues made it difficult to make unequivocal assignments.

The $tRNA^{Trp}$ fragment that bound to the polymerase did not hybridize to the 35S RNA genome because parts of the 3' oligonucleotide tract required for binding to the viral genome was absent from this fragment. A recent report concluded that essentially the entire $tRNA^{Trp}$ was required for binding

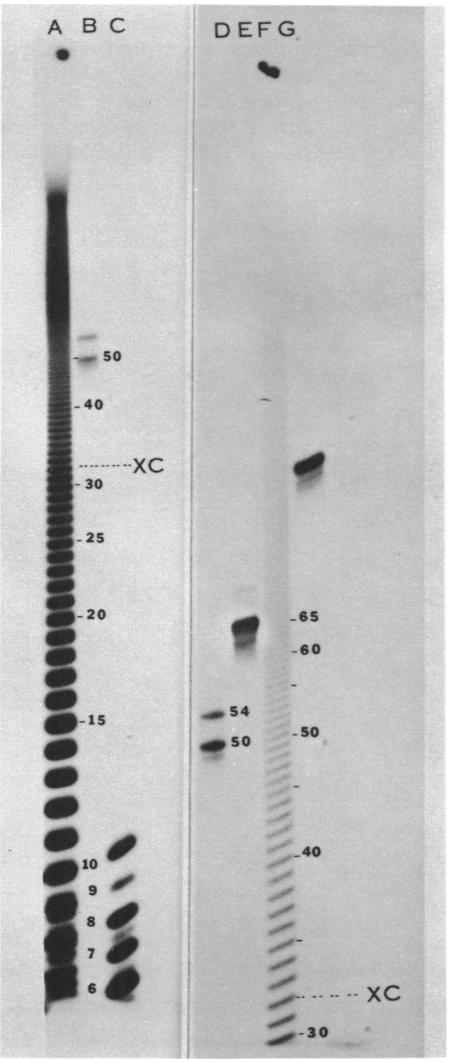

Fig. 3. Sequence assignment for the major $tRNA^{Trp}$ fragment that binds to AMV DNA polymerase. The major $tRNA^{Trp}$ fragment was extrapolated to be 67 residues on the basis of its mobility on a 20% denaturing polyacrylamide gel (Fig. 3E). Poly rA markers were used to calibrate the gel (3F). Markers used were previously standardized by oligonucleotides of known lengths $A(-A)_{65}$ to $A(-A)_9$ (3A and 3C). A tRNA fragment 54 nucleotides long was used as a secondary marker for the longer fractionation when the synthetic oligonucleotides eluted from the gel (3B and 3D).

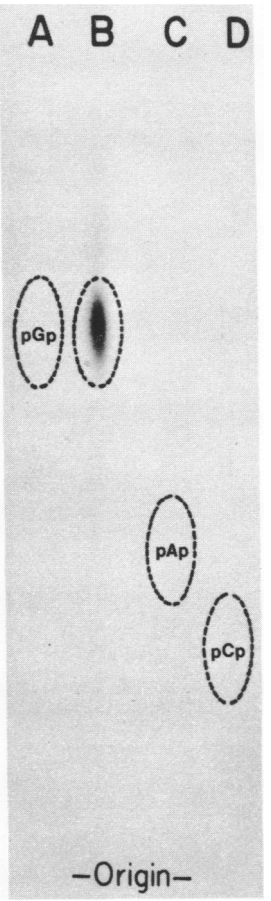

Fig. 4. Analysis of the 5'-end nucleotide of the [^{32}P] $tRNA^{Trp}$ fragment. The end-labeled nucleotide diphosphate obtained by alkaline hydrolysis of 5'-^{32}P$tRNA^{Trp}$ fragment was fractionated on 3 MM Whatman paper and analyzed by electrophoresis (Fig. 4B) with authentic markers. The 3 MM Whatman paper was equilibrated with 50 mM formate-(NH_4^+) (pH 3.5), containing 1 mM EDTA.

to the enzyme (11). Our findings did not assign a critical role to the 3'-stem as a site for binding. The association between the ψψCG loop and the dihydrouridine loop can be maintained by the $tRNA^{Trp}$ fragment and appears to be required for interaction with the enzyme.

It is attractive to postulate that AMV DNA polymerase binds with $tRNA^{Trp}$ by interacting with elements of its secondary and tertiary structure. While associated, the polymerase alters the topology of $tRNA^{Trp}$ to permit its interaction with the viral 35S genome. The precise function, if any, of the AMV DNA polymerase associated nuclease (19,20) in the processing of the tRNA primer remains to be determined.

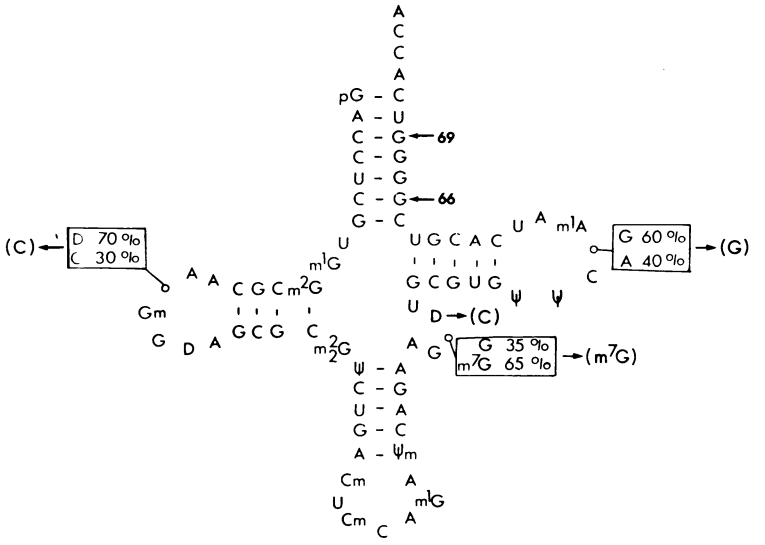

Fig. 5. Primary structure of $tRNA^{Trp}$ from bovine liver. Nucleotides in brackets are those present in $tRNA^{Trp}$ (avian). Boxed nucleotides show the percent distribution of nucleotides at the particular site in $tRNA^{Trp}$ (bovine).

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REFERENCES

1. Taylor, J.M. and Illmensee, R. (1975) J. Virol. 16, 553-558.
2. Verman, I.H., Meuth, N.L., Bromfeld, E., Manly, K.F. and Baltimore, D. (1971) Nature New Biol. 233, 131-134.
3. Dahlberg, J.E., Sawyer, R.C., Taylor, J.M., Faras, A.J., Levinson, W.E., Goodman, H.M. and Bishop, J.M. (1974) J. Virol. 13, 1126-1133.
4. Canaani, E. and Duesberg, P.H. (1972) J. Virol. 10, 23-31.
5. Sawyer, R.C. and Dahlberg, J.E. (1973) J. Virol. 12, 1226-1237.
6. Baroudy, B.M., Fournier, M., Labouesse, J., Papas, T.S. and Chirikjian, J.G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1889-1893.
7. Cordell, B., Stavnezer, E., Friedrich, R., Bishop, J.M. and Goodman, H.M. (1976) J. Virol. 19, 548-558.
8. Harada, F. and Dahlberg, J.E. (1975) Nucleic Acids Res. 2, 865-871.

9. Eiden, J.J., Quade, K. and Nichols, J.L. (1976) Nature 259, 245-247.
10. Panet, A., Haseltine, W.A., Baltimore, D., Peters, G., Harada, F. and Dahlberg, J.E. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2535-2539.
11. Cordell, B., Swanstrom, R., Goodman, H.M. and Bishop, J.M. (1979) J. Biol. Chem. 254, 1866-1874.
12. Chirikjian, J.G., Rye, L. and Papas, T.S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1142-1146.
13. Papas, T.S., Chirigos, M.A. and Chirikjian, J.G. (1974) Nucleic Acids Res. 1, 1399-1409.
14. Papas, T.S., Marciani, D.J., Samuel, K. and Chirikjian, J.G. (1976) J. Virol. 18, 904-910.
15. Baroudy, B.M., Chirikjian, J.G. (1979) International Congress of Biochemistry, Toronto, Canada.
16. Fournier, M., Dorizzi, M., Sarger, C. and Labouesse, J. (1976) Biochemie 58, 1159-1167.
17. Donis-Keller, H., Maxam, A.M. and Gilbert, W. (1977) Nucleic Acids Res. 4, 2527-3538.
18. Topal, M.D. and Fresco, J.R. (1976) Nature 263, 289-293.
19. Samuel, K., Papas, T.S. and Chirikjian, J.G. (1979) Proc. Natl. Acad. 76, 2659-2663.
20. Golomb, M. and Grandgenett, D.P. (1979) J. Biol. Chem. 254, 1606-1613.