Volume 12 Number 5 1984 Nucleic Acids Research

Interactions between avian myeloblastosis reverse transcriptase and $tRNA^{Trp}$. Mapping of complexed tRNA with chemicals and nucleases

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Received 13 January 1984; Accepted 15 February 1984

ABSTRACT

The interactions between beef $tRNA^{Trp}$ with avian myeloblastosis reverse transcriptase have been studied by statistical chemical modifications of phosphate (ethylnitrosourea) and cytidine (dimethyl sulfate) residues, as well as by digestion of complexed tRNA by Cobra venom nuclease and Neurospora crassa endonuclease. Results with nucleases and chemicals show that reverse transcriptase interacts preferentially with the D arm, the anticodon stem and the Tψ stem. All these regions are located in the outside of the L-shaped structure of tRNA. This domain of interaction is different to that reported previously in the complex of beef tRNA with the cognate aminoacyl-tRNA synthetase (M. Garret et al.; Eur. J. Biochem. In press). Avian reverse transcriptase destabilizes the region of tRNA where most of the tertiary interactions maintaining the structure of tRNA are located.

INTRODUCTION

Avian myeloblastosis reverse transcriptase (RNA-dependent DNA polymerase [E.C. 2.7.7.7]) synthesizes a complementary DNA (cDNA) copy of the viral RNA genome using $tRNA^{Trp}$ as primer (1-3). Primer $tRNA^{Trp}$ is encapsidated in the virion and found partially base paired to the 35 S viral RNA. The interaction occurs between 16 bases from the 3' end of tRNA and a complementary sequence near the 5' end of the viral genome. DNA synthesis is thus started from the terminal adenosine of primer tRNA. Several lines of evidences suggest strongly that reverse transcriptase may be involved in the base pairing of primer tRNA to the viral genome: (i) it was found that the annealing of $tRNA^{Trp}$ to avian myeloblastosis virus (AMV) RNA is mediated by avian reverse transcriptase which catalyzes the partial unwinding of the acceptor stem of $tRNA^{Trp}$ (4); (ii) temperature sensitive mutants of reverse transcriptase are unable to bind primer tRNA (5); (iii) mutants of avian sarcoma virus lacking a functional reverse transcriptase were found to have a genomic RNA lacking primer $tRNA^{Trp}$ (6); (iv) reverse transcriptase has an increased affinity for its primer tRNA as compared with the other cellular tRNAs (6). These observations led to study the interactions between these two macromolecules in order to look for the regions in

the primer molecule specifically recognized by the polymerase. Using the UV induced crosslinking between AMV reverse transcriptase and $tRNA^{Trp}$ it was found that reverse transcriptase interacts closely with the anticodon region and the CCA end of $tRNA^{Trp}$ (7). By affinity labelling of avian reverse transcriptase by periodate oxidated $tRNA^{Trp}$, the CCA end of tRNA was found covalently linked to the α subunit of reverse transcriptase which is the catalytic core of the polymerase (8). Recently it was shown that loop IV of $tRNA^{Trp}$ containing the unusual sequence GψψC was important in binding to reverse transcriptase (9).

In this communication we show the results of experiments designed to study the interaction areas of primer $tRNA^{Trp}$ with reverse transcriptase by statistical chemical phosphate and base modification experiments and statistical nuclease digestions of the nucleic acid. This approach was already successfully used for studying complexes between tRNAs and their cognate aminoacyl-tRNA synthetases or elongation factor (10-14) and recently it allowed to probe the tertiary structure of $tRNA^{Trp}$ in solution, as well as the regions of this tRNA recognized by the cognate tryptophanyl-tRNA synthetase (15).

MATERIALS AND METHODS

Avian myeloblastosis virus reverse transcriptase, purified as described in (16), was obtained from Dr. J. W. Beard (Life Sciences Inc. Florida. USA). The enzyme was homogeneous as judged by polyacrylamide electrophoresis in the presence of SDS; it is a dimeric protein of the αβ type (60 Kd and 90 Kd respectively). Its specific activity is 30,400 Units/mg. Beef liver $tRNA^{Trp}$ and tryptophanyl-tRNA synthetase were purified as described previously (17, 18). It is recalled that the sequence of beef liver $tRNA^{Trp}$ is nearly identical to that of chicken $tRNA^{Trp}$ (19). Yeast valyl-tRNA synthetase and tRNA nucleotidyl transferase were purified to homogeneity as previously described (20, 21). Radioisotopes, (α-^{32}P)ATP 550 Ci/mmole and (γ-^{32}P)ATP 5,000 Ci/mmole, were from New England Nuclear (FRG). Ethylnitrosourea was purchased from Fluka, Switzerland. Alkaline phosphatase and venom phosphodiesterase were from Worthington. Acrylamide and bis-acrylamide were from BDH Chemicals Ltd. Poole, England. Dimethyl sulfate was from Aldrich. Ribonuclease T_1 from Sigma Chem. Co. Neurospora crassa endonuclease was purchased from Boehringer Mannheim. Ribonuclease from cobra Naja oxiana venom (EC. 3.1.26.3) was purified as described before (11). Polynucleotide kinase was purchased from BRL- (FRG).

End-labelling of $tRNA^{Trp}$.

Beef liver $tRNA^{Trp}$ was labelled in the terminal adenosine by using (α-

^{32}P)ATP and tRNA nucleotidyl transferase and in the 5' end with (γ-^{32}P)ATP and polynucleotide kinase essentially as described in a previous article (15).

Alkylation of complexed $tRNA^{Trp}$ by ethylnitrosourea.

The method of alkylation of the complex between $tRNA^{Trp}$ and the homologous or heterologous aminoacyl-tRNA synthetase has been described before (15). The concentration of tRNA labelled in the 5' or 3' end was 0.5 µM, while tryptophanyl-tRNA synthetase from beef pancreas or yeast valyl-tRNA synthetase was 1.5 µM. Other experimental details are given in (22, 23).

For the experiments involving the complex between $tRNA^{Trp}$ and reverse transcriptase, the enzyme was dialyzed just before the incubation with tRNA against a buffer 50 mM Tris-HCl pH 8, 2 mM DTE, 0.2% Triton X-100 and 50% glycerol. The incubation mixture for the complex between reverse transcriptase and tRNA contained in a final volume of 20 µl: 50 mM Tris-HCl pH 8, 40 mM KCl, 0.3 mM EDTA, 6 mM $MgCl_2$, 1 mM DTE, 0.1% Triton X-100, 25% glycerol, 0.25 or 0.50 µM of 3' or 5' end labelled $tRNA^{Trp}$ and 0.85 µM AMV reverse transcriptase. The enzyme concentration was determined by the fluorescamine method (24). Alkylation of the complex was performed as described before (15, 25). No changes in the alkylation pattern of native $tRNA^{Trp}$ were observed when the buffer described in (15) was replaced by the reverse transcriptase stabilizing buffer described above (16).

Alkylation of the N_3 positions of cytidines in $tRNA^{Trp}$ by dimethyl sulfate.

Before utilisation reverse transcriptase was dialyzed as in the previous paragraph. The incubation mixture was as above. Dimethyl sulfate was used at a final concentration of 0.25% (v/v). Other conditions were as described by Peattie and Gilbert (26) and in our previous article (15). Control experiments performed with ethylnitrosourea and dimethyl sulfate showed that the enzyme was still active after incubation with the reagent (12,200 and 15,200 Units/mg respectively).

Partial hydrolysis by nuclease digestion of the tRNA-enzyme complex using cobra Naja oxiana venom nuclease and Neurospora crassa endonuclease.

The incubation mixtures were as follows: i) Cobra venom nuclease: In a final volume of 0.01 ml the mixture contained, 50 mM KH_2PO_4 pH 7.2, 0.5 mM DTE, 10 mM $MgCl_2$, 0.05% Triton X-100, 12.5% glycerol, 0.42 µM AMV reverse transcriptase, 0.2-0.4 µM tRNA labelled at the 3' or 5' end and 0.005 to 0.01 units of Cobra venom nuclease. Incubation was carried out at 0°C for 1 or 5 min. The reaction was stopped by precipitation with ethanol, the pellet was dried and submitted to polyacrylamide gel electrophoresis. ii) Neurospora cras-

sa endonuclease: The incubation mixture contained, 50 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 0.5 mM DTE, 0.05% Triton X-100, 12.5% glycerol, 0.42 µM reverse transcriptase, 0.15-0.30 µM $tRNA^{Trp}$ and 0.25 units of endonuclease. Incubation was carried out at 37°C for 5 min.

Analysis of splitting products and assignment of electrophoretic bands.

The oligoribonucleotides liberated after chain-scission of the alkylated $tRNA^{Trp}$ or after nuclease digestion of the same nucleic acid were analyzed on polyacrylamide gels. Assignment of electrophoretic band was done by comparison with partial formamide and ribonuclease T_1 digests (27, 28).

RESULTS AND DISCUSSION

Alkylation of tRNA-reverse transcriptase complex by ethylnitrosourea.

End-labelled $tRNA^{Trp}$ was alkylated with ethylnitrosourea in the presence or absence of AMV reverse transcriptase. The protection of some regions of tRNA against alkylation when reverse transcriptase was present can be visualized in the results shown in Figure 1. The intensity of the autoradiographic bands in positions 22 - 23 and 42 -45 are clearly diminished in the lane corresponding to the enzyme-tRNA complex (Fig. 1A, B). Less dramatic protections were seen in positions 51 and 52 (Fig. 1C). It can be seen that some spontaneous degradation bands are increased in the presence of reverse transcriptase alone (lane 6). The effect is particularly pronounced at position 36 and to a lower extent at positions 21, 14 and 9. This probably reflects some conformational changes of tRNA induced when the retroviral polymerase is bound to primer tRNA. Concerning the strong cut at position 36 in the anticodon loop (see Lane 6 Fig. 1) it was verified that it is not due to nuclease contamination. Indeed, when other tRNAs than $tRNA^{Trp}$ (i.e. labelled yeast $tRNA^{Phe}$) is added with the enzyme no additional band appeared in the anticodon region (not shown). Fig. 2 shows the example of the densitometric profile of an autoradiogram (Fig. 1A).

All results are summarized in Fig. 3A. It can be seen that the regions protected against alkylation in the complex correspond to the D-arm, the anticodon arm, the variable loop and the Tψ arm. Thus the interactions leading to protection of some regions of tRNA against alkylation can be found in the outer region of the L-shaped structure commonly ascribed to tRNA. This is in sharp contrast with results found when studying the interactions between tRNA and the cognate aminoacyl-tRNA synthetases (29). In the case of the interactions between beef $tRNA^{Trp}$ and tryptophanyl-tRNA synthetase studied using the same approach (15), it was shown that the $tRNA^{Trp}$ regions protected against ethylni-

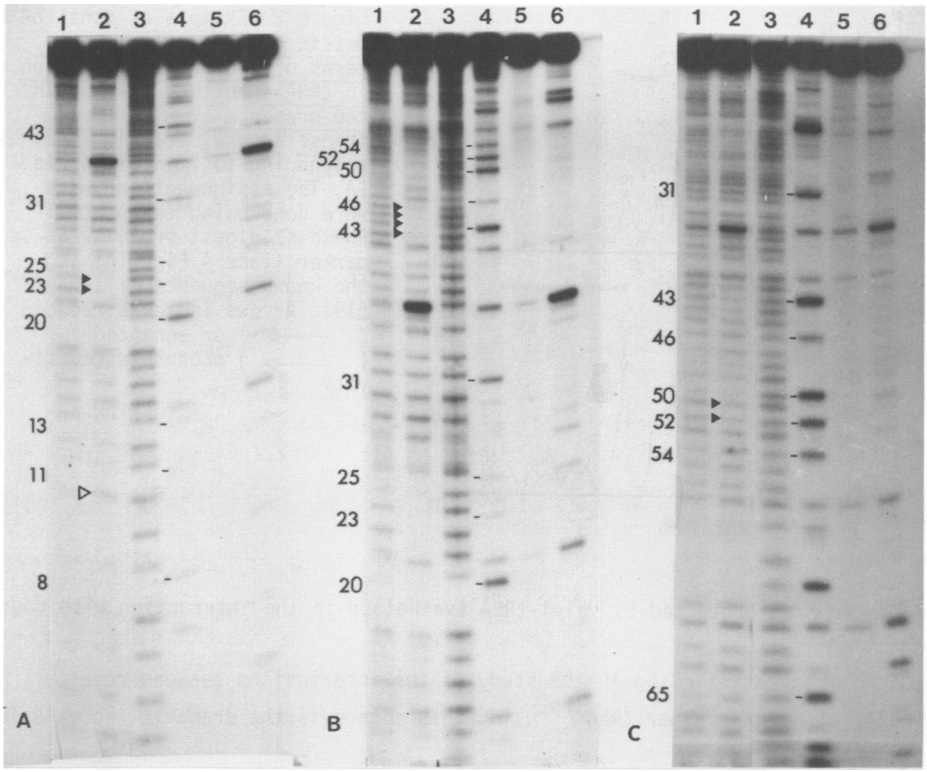

Figure 1. Autoradiography of polyacrylamide gels after phosphate alkylation experiments with 5' labelled (A, B) and 3' labelled $tRNA^{Trp}$ (C) in the presence of reverse transcriptase. Analysis was performed on 20% (A) and 15% (B, C) acrylamide. (1) Alkylation in the absence of enzyme; (2)alkylation in the presence of reverse transcriptase; (3) alkylation under conditions unfolding the tRNA structure; (4) partial RNase T_1 digestion; (5,6) control incubation of tRNA in the absence of ethylnitrosourea; (5) no enzyme; (6) plus reverse transcriptase. The nucleotide numbering was derived from the one of yeast $tRNA^{Phe}$ (40). Note that position 17 is missing. Guanosine residues are numbered according to phosphates (22). ▶ Phosphates protected by reverse transcriptase; ▷ phosphate exhibiting an increased reactivity in the presence of the enzyme.

trosourea alkylation are found inside the L-shaped structure. In Fig. 3B, C is also shown the alkylation pattern of phosphate in $tRNA^{Trp}$ complexed with tryptophanyl-tRNA synthetase and valyl-tRNA synthetase. The regions in $tRNA^{Trp}$ protected by reverse transcriptase and the tryptophanyl-tRNA synthetase are clearly different. Under the same experimental conditions used for tryptophanyl-tRNA synthetase, the level of phosphate protection by valyl-tRNA synthetase is weaker except in the extra loop region. It is interesting that the latter

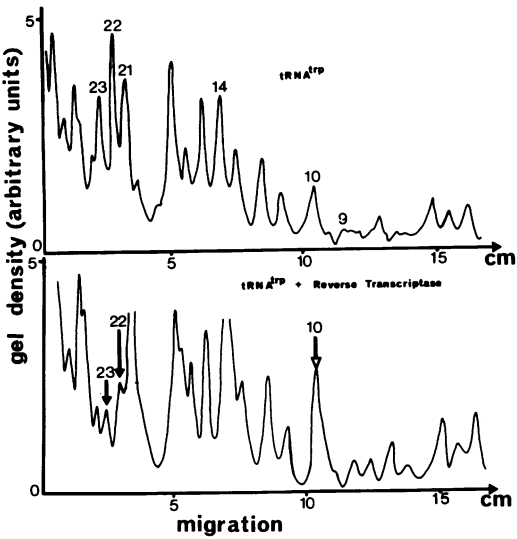

Figure 2. Example of densitometric tracings of the patterns of phosphate alkylation in $tRNA^{Trp}$ either free or in the presence of AMV reverse transcriptase. Tracings corresponding to 1 and 2 in Fig. 1A. The assignments of pics were done using the partial RNase T1 digest products as marker (lane 4 Fig. 1A) and the known sequence of $tRNA^{Trp}$ (19). Arrows indicate reduced (⟶) or enhanced (⇢) phosphate reactivity.

region is also protected by valyl-tRNA synthetase in the interaction with cognate $tRNA^{Val}$ (10).

An important finding in the study of the interactions between reverse transcriptase and primer $tRNA^{Trp}$ using this method is the dramatic increase in

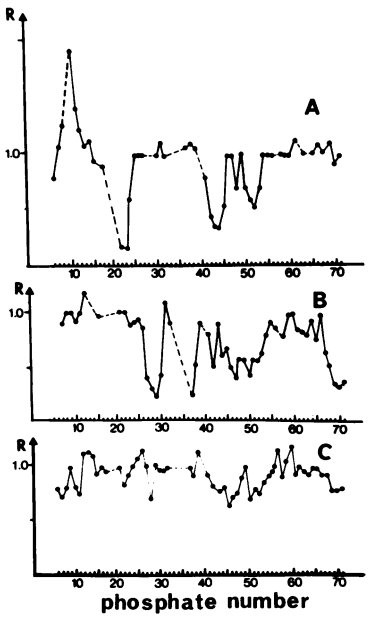

Figure 3. Patterns of phosphate alkylation by ethylnitrosourea in the complexes between $tRNA^{Trp}$ and the indicated enzymes. R values are the ratio between the intensities of the corresponding electrophoretic bands of the alkylated $tRNA^{Trp}$ in the presence of enzymes or alone. A ratio R < 1 means that the alkylation of a given phosphate is lower in the presence of the enzyme. The dotted lines join phosphate residues adjacent to non-measurable phosphates. The reactivities of phosphates 1-6 and 72-76 was not measured as explained in the text. Experimental data for aminoacyl-tRNA synthetase are from (15). A: reverse transcriptase; B: beef tryptophanyl-tRNA synthetase; C: yeast valyl-tRNA synthetase.

reactivity of phosphate in position 10 observed when tRNA is alkylated in the presence of reverse transcriptase. Phosphate 10, as well as the proximal phosphate 9 are protected against alkylation by ethylnitrosourea in the native structure of $tRNA^{Trp}$ (15), a fact which is also found in other tRNAs (22, and Romby et al., manuscript in preparation). This finding may reflect a structural similarity between all tRNAs at this level. This protection is well explained by the crystal structures of $tRNA^{Phe}$ (30) and $tRNA^{Asp}$(31) and can be related to the proximity of nucleotides 9 and 10 with the variable loop. For instance, in the M.I.T. model of $tRNA^{Phe}$ the oxygen atom O_1 of phosphate 10 interacts with the 2' OH of ribose 45 (32) and then helps to stabilize the junctions between the D and anticodon arms. The low reactivity of phosphate 10 is also supported by computations showing, in the case of yeast $tRNA^{Phe}$, a steric inaccessibility to external probes (33, 34). The increased reactivity of phosphate 10 we have found, thus implies an unshielding of the residue consecutive to a structural rearangement of tRNA induced by reverse transcriptase.

As shown previously, reverse transcriptase is probably involved in the positioning of primer $tRNA^{Trp}$ on the viral genome through the partial unwinding of the acceptor stem of $tRNA^{Trp}$ (3, 4). The destabilizing effect observed at the level of the sharp bend between residues 9 and 10 of $tRNA^{Trp}$ in the presence of reverse transcriptase supports strongly this idea, since the loosening of the interaction between the sharp bend and the variable loop can be the primary event in the unwinding of the Tψ arm and the acceptor stem of tRNA. This is a compulsory step before the annealing of the 3' end of tRNA to the complementary sequence in the viral genome.

It is important to point out, concerning this approach, that small fragments of tRNA are lost during ethanol precipitation after the modified phosphates have been hydrolyzed. For this reason no interactions with the 5' or 3' ends of $tRNA^{Trp}$ can be tested using this method.

Accessibility of cytosine residues in $tRNA^{Trp}$ complexed with AMV reverse transcriptase.

Beef $tRNA^{Trp}$ modified with dimethyl sulfate was incubated alone or with the retroviral polymerase as described in Methods.

Cytosine residues of $tRNA^{Trp}$ in the 3' terminal CCA and in the anticodon are accessible to external probes in the native structure of $tRNA^{Trp}$ (15, 35). In this experiment we have probed the accessibility of these cytosines toward dimethyl sulfate in the presence of reverse transcriptase. Cytosine residues of the anticodon region have been shown to play a role in the aminoacylation process of this tRNA (15, 35).

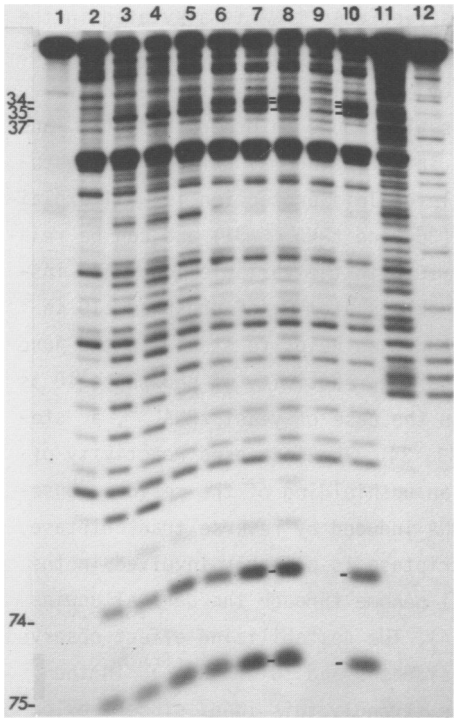

Figure 4. Autoradiogram of 20% acrylamide gels after cytidine alkylation with dimethyl sulfate of 3' labelled tRNA either free or in the presence of AMV reverse transcriptase. (1) Control without incubation; (2) control incubation without reagent; (9) control incubation without reagent plus reverse transcriptase; (3) treatment with reagent for 30 sec under denaturing conditions; (4) as in (3), but for 60 sec; (5) treatment under semidenaturing conditions; (6, 8) treatment under native conditions in the absence of enzyme; (7, 10) treatment under native conditions in the presence of reverse transcriptase. The treatment with dimethyl sulfate was done for 5 min (6-7) or 12 min (8,10); (11) formamide ladder; (12) partial RNase T_1 digest. In this case numbering corresponds to base position.

As seen in Fig. 4 these residues (as m^1G37 which is cut in our conditions by the treatment (15)) are not protected against dimethyl sulfate in the presence of AMV reverse transcriptase. Moreover, none of the N_3 atoms of the cytidines protected in the secondary structure of tRNA against dimethyl sulfate becomes accessible in the presence of the enzyme. The only tertiary interaction tested with this approach (C57-G18) did not show any appreciable difference in the presence or absence of polymerase. In a previous study using UV induced crosslinking of tRNA and reverse transcriptase, it was shown that both the anticodon region and the 3' end of primer tRNA were in close interaction with the enzyme (7). Using the method described in this article we can give a more precise detail of these interactions and dismiss cytidines 74 and 75, as well as those in the anticodon loop as being in close contact with the enzyme in the reverse transcriptase-tRNA complex.

Partial digestion of free $tRNA^{Trp}$ and reverse transcriptase-$tRNA^{Trp}$ complex by Cobra venom nuclease and Neurospora crassa endonuclease.

We have used two nucleases to study the interactions between the retroviral polymerase and its specific primer. Cobra venom nuclease is highly specific

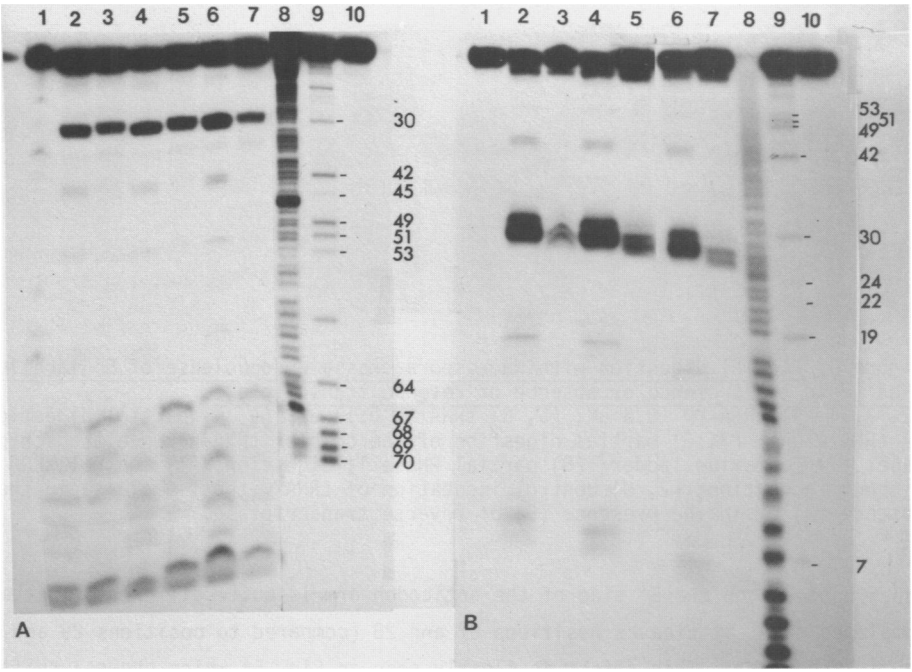

Figure 5. Partial digestion with Cobra venom nuclease of 3' end labelled (A) and 5' labelled $tRNA^{Trp}$ (B) in the absence or presence of AMV reverse transcriptase. Analysis was done in 15% polyacrylamide gels. (1) Control experiment in the absence of nuclease plus reverse transcriptase;(2, 3, 4, 5) partial digestions with 0.01 units of nuclease in the absence (2, 4) or in the presence (3, 5) of reverse transcriptase. Incubation was performed for 1 min (2, 3) or 5 min (4, 5); (6, 7) incubations for 1 min with 0.005 units of nuclease in the absence (6) or in the presence of reverse transcriptase (7); (8) formamide ladder; (9) partial ribonuclease T1 digestion; (10) control treatment in the absence of both nuclease and polymerase.

of double stranded regions (36), while the N. crassa endonuclease under the conditions used in our experiments (low ionic strength) has no specificity concerning the structure or sequence of the target (37, 38). Using free $tRNA^{Trp}$ both nucleases attack preferentially the anticodon region of tRNA. As seen in Fig. 5 Cobra venom nuclease preferentially cleaves the 5' part of the anticodon arm in positions 28-29. Weaker cuts are found in the 3' side of this arm at positions 41-42. Other cuts are found at the 3' terminus of the tRNA (Fig. 5A) and around position 7 and 19 (Fig. 5B); these last cuts are of secondary origin, since they appear only with tRNA labelled at the 5' end (12). In the presence of reverse transcriptase positions 41-42 become completely inaccessible to this nuclease (see lanes 3, 5, 7 Fig. 5). The effect of the

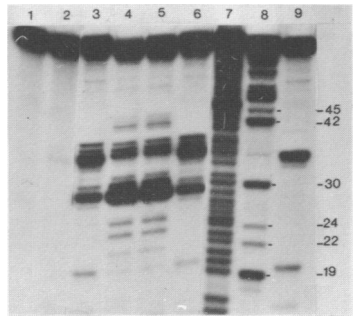

Figure 6. Partial digestion with Neurospora crassa endonuclease of 5' labelled $tRNA^{Trp}$ in the presence or absence of reverse transcriptase.
(2, 3, 4, 9) $tRNA^{Trp}$ 0.3 µM; (5, 6) $tRNA^{Trp}$ 0.15 µM; (4, 5) partial digestion of tRNA alone; (3, 6) partial digestion of the complex tRNA-reverse transcriptase; (7) formamide ladder; (8) partial RNase T_1 digestion; (1) control tRNA without incubation; (2, 9) control incubation of tRNA without nuclease in the absence (2) or in the presence (9) of reverse transcriptase.

Cobra nuclease on the 5' side of the anticodon arm is quite different. In the complexed tRNA it cleaves positions 27 and 28 (compared to positions 29 and 28 in free tRNA). This effect is clearly seen in Fig. 5A which shows a shift in migration for the doublet in free or complexed tRNA. The effect appears in a more quantitative way in Fig. 5B where the disappearence of cleavage at position 29 and the decrease of that at position 28 are clearly seen. The new cut considered above at position 27 is best seen in lane 7 (Fig. 5B).

Experiments conducted with Neurospora crassa endonuclease essentially confirm the results obtained with dimethyl sulfate and Cobra venom nuclease (Fig. 6). The three cuts in the anticodon loop in positions 35, 36, 37 are not suppressed in the presence of reverse transcriptase. Considering the anticodon stem region, protections appear at positions 40, 41 and 29, 30 (lanes 3, 6 Fig. 6). Using Neurospora crassa endonuclease and Cobra venom nuclease no protections in the complexed tRNA are found at the 3' terminus nucleic acid as previous seen in (4).

CONCLUSIONS

Two types of evidences arise from the present study: (i) AMV reverse transcriptase interacts with the anticodon stem, the 3' side of the D arm and the 5' side of the TΨ stem of beef $tRNA^{Trp}$. The present data agree with previous results (9, 39). Thus, the existence of protection in the anticodon stem region was shown by UV crosslinking experiments (7). (No protection how-

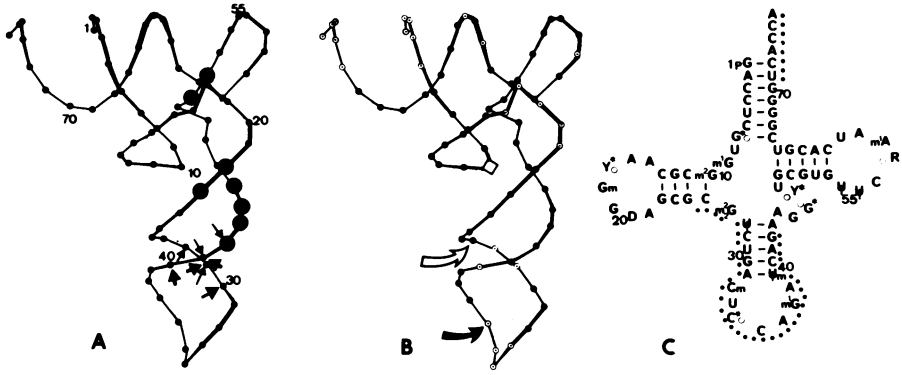

Figure 7. Diagramatic representation of the interactions of $tRNA^{Trp}$ with AMV reverse transcriptase using the approaches described in this work.
We have assumed for beef $tRNA^{Trp}$ a tertiary structure identical to that of $tRNA^{Phe}$ from yeast. The ORTEP drawing was adapted from (10). A: ● phosphates protected by the enzyme against alkylation. Position protected against Cobra venom nuclease (⟶) and Neurospora crassa endonuclease (➔) by reverse transcriptase. B: positions where a conformational change is observed in the presence of reverse transcriptase. (◇) phosphate reactivity is enhanced, (⇨) accessibility towards Cobra venom nuclease is enhanced, (➘) spontaneous degradation is enhanced, (⊙) phosphates not tested. C: cloverleaf structure of $tRNA^{Trp}$ (19) showing the regions involved in UV-induced crosslinked complex with reverse transcriptase (7). Open circles represent heterogeneities, R stands for purine, Y for pyrimidine and an asterisk for a modified base. Numbering corresponds to phosphate position.

ever, could be detected in the 3' terminal quarter of the molecule, which has to unwind to anneal to the viral RNA, nor in the anticodon loop). This is in agreement with Baroudy and Chirikjian (39) who showed that $tRNA^{Trp}$ molecules lacking the last 8 nucleotides at the 3' end are able to bind to the reverse transcriptase. Moreover, recent results of Hu and Dahlberg show that the anticodon loop does not represent a structural requirement for the interaction, since cleaved molecules in that region do bind stably to reverse transcriptase (9). (ii) Three lines of evidence point out for conformational changes in tRNA induced by reverse transcriptase. Firstly, the strong spontaneous cut which specifically appears in the anticodon of $tRNA^{Trp}$ supports a rearrangement of the conformation around this residue upon complex formation. This is a long range effect, since this region is not involved in direct interaction with the polymerase. A similar explanation could account for the observed involvement of the $\psi_{54}\psi_{55}$ residues in the interaction process with reverse transcriptase (9), since according to our data these residues are not in contact with the protein. Secondly, Cobra venom experiments reveal the emergence

of a new cut in position 27. Ethylnitrosourea mapping exhibits an increased reactivity of phosphate 10 in complexed tRNA. These two last residues are located in the central part of tRNA where the two branches of the L join to form the characteristic three dimensional structure of tRNA (Fig. 7). Most of the tertiary interactions found in tRNA are confined to this region. The present data clearly indicate that reverse transcriptase destabilizes this crucial part of the molecule. We propose that this destabilization represents the first step towards the unwinding of this region of tRNA complementary to the viral genome.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Centre National de la Recherche Scientifique (CNRS), the University of Bordeaux II, NATO and the Fondation pour la Recherche Médicale. The authors are grateful to Drs. J.P. Ebel and B. Labouesse for stimulating discussion.

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