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A model for the tertiary structure of mammalian mitochondrial transfer RNAs lacking the entire 'dihydrouridine' loop and stem

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The mammalian mitochondrial $tRNA^{Ser}_{AGY}$ is unique in lacking the entire dihydrouridine arm. This reduces its secondary structure to a 'truncated cloverleaf'. Experimental evidence on the tertiary structure has been obtained by chemically probing the conformation of both the bovine and human species in their native conformation and at various stages of denaturation. A structural model of the bovine tRNA is presented based on the results of this chemical probing, on a comparison between nine homologous 'truncated cloverleaf' secondary structures and on analogies with the crystal structure of yeast phenylalanine tRNA. The proposed structure is very similar in shape to that of yeast $tRNA^{Phe}$ but is slightly smaller in size. It is defined by a unique set of tertiary interactions. Structural considerations suggest that other mammalian mitochondrial tRNAs have smaller dimensions as well.

Key words: mitochondrial tRNA/tRNA structure/chemical probing

Introduction

Through sequence analysis of mammalian mitochondrial (mt) genomes, 22 tRNA genes have been identified (Anderson *et al.*, 1981, 1982a; Bibb *et al.*, 1981). The encoded tRNAs are thought to be sufficient in number to read the mammalian mt genetic code, with the possible exception of the methionine codons, using a unique pattern of codon recognition (Barrell *et al.*, 1980). They are unusual in lacking many of the invariant and semi-invariant features (Sprinzl and Gauss, 1982a, 1982b; Dirheimer *et al.*, 1979; Clark and Klug, 1975) of non-mitochondrial tRNAs. Although all but one can form the familiar cloverleaf secondary structure, other features are absent, such as the constant seven-nucleotide size of the TψC loop, the sequence T-ψ-C-R-A and a number of conserved bases in the D loop and elsewhere. The one exception is the $tRNA^{Ser}_{AGY}$ which lacks the entire D arm (de Bruijn *et al.*, 1980; Acari and Brownlee, 1980) and whose secondary structure is reduced to a 'truncated cloverleaf' (c.f. Figure 2). Nevertheless, this tRNA is presumed to function in mt protein synthesis. It can be specifically aminoacylated with serine and the corresponding codons, which occur in almost all identified mammalian mt protein-coding genes, specify serine (de Bruijn *et al.*, 1980).

If this assumption is correct, then the question arises as to how $tRNA^{Ser}_{AGY}$ folds spatially into a three-dimensional structure enabling serine to be placed in the correct position on the ribosome for chain elongation. It is addressed by chemically probing two species of mt $tRNA^{Ser}_{AGY}$, bovine and human, in their native conformation and at various stages of denaturation. The results are used to help construct a tertiary structure model of $tRNA^{Ser}_{AGY}$, based also on a comparison of $tRNA^{Ser}_{AGY}$ secondary stuctures from nine different mammalian species and on tertiary structure elements of the crystal structure yeast $tRNA^{Phe}$. The implications of the proposed structural model for other mammalian mitochondrial tRNAs are discussed.

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Results and Discussion

Specificity of the chemical probes

Chemical probing of RNA, according to Peattie and Gilbert (1980), relies on three chemical modification reactions. Dimethylsulphate (DMS) monitors the availability of N-7 in guanosines and N-3 in cytidines by methylation and diethylpyrocarbonate (DEP) monitors availability of N-7 in adenosines by carbethoxylation. These reactions lead to exposure of the corresponding ribose ring providing a specific site for strand scission by aniline. If the initial modifications by DMS and DEP are limited in extent and all subsequent reactions complete, a nested set of fragments is obtained with a common 3′ terminus. By labeling the RNA with [5′-^{32}P]pCp before probing and fractionating the fragments resulting from strand scission on a polyacrylamide gel, a sequence pattern is produced punctuated by gaps or weak bands representing the bases that were unavailable for modification.

N-3 positions in cytidines are involved in Watson-Crick hydrogen bonding. Consequently, G.C base pairs are monitored but also reverse Watson-Crick base pairs such as G_{15}-C_{48} in yeast $tRNA^{Phe}$ (Jack *et al.*, 1976; Peattie and Gilbert, 1980). N-7 positions in guanosines are involved in weaker tertiary interactions, such as G_{22} in the $G_{22}-G_{46}$ connection of the yeast $tRNA^{Phe}$ structure (Jack *et al.*, 1976) and in coordination interactions with various ions or water molecules (Jack *et al.*, 1977; Holbrook *et al.*, 1977; Hingerty *et al.*, 1978). N-7 positions in adenosines are involved in the reverse Hoogsteen base pairs T_{54}-m^1A_{58} and U_8-A_{14} of yeast $tRNA^{Phe}$ as well as in the base triple A_9-U_{12}-A_{23} (Jack *et al.*, 1976). DEP has also been found to monitor stacking of adenosines in yeast $tRNA^{Phe}$ (Peattie and Gilbert, 1980).

Choice of solution variables

The conformation of a tRNA in solution is sensitive to variables such as pH, salt concentration and temperature (Cole *et al.*, 1972; Bina-Stein and Crothers, 1974). In acidic conditions tRNA loses its native conformation due to protonation. In low salt, tRNA tertiary structure is greatly destabilized because lowering the ionic strength reduces electrostatic shielding of the negatively charged phosphate groups in the RNA backbone. This results in loss of native structure and transition to a conformation with reduced free energy (Crothers, 1979; Potts *et al.*, 1979). In high salt, at neutral pH and low temperatures the tRNA conformation is considered native.

Mg^{2+} ions greatly stabilize tertiary structure and this is not just due to shielding of phosphate groups but to binding of

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Mg^{2+} at specific sites (Jack *et al.*, 1977; Hingerty *et al.*, 1978). In terms of melting behaviour its effect is to increase significantly the temperature at which tertiary structure is lost (Cole *et al.*, 1972; Crothers, 1979; Potts *et al.*, 1979). The mammalian $tRNA^{Ser}_{AGY}$ species have been probed under two buffer conditions, namely 50 mM sodium cacodylate (pH 7.5), 120 mM NaCl and either 1 mM EDTA or 1 mM $MgCl_2$. This satisfies both the requirements for stable tertiary structure (Crothers *et al.*, 1974) and those for performing the chemical probing reactions (Peattie and Gilbert, 1980).

We have not had enough material to determine the melting curve of the molecule optically, but by carrying out the chemical probing at a series of 5° intervals between 5 and 90°C (Figure 1), we have effectively replaced it. Moreover, the information gained is much more extensive since this approach allows one to follow not just the melting of the tertiary and secondary structures, but that of individual base pairs, something not easily done by physical methods. It will be seen from Figure 1 that the secondary structure, in the absence of Mg^{2+}, melts out on the average at ~40°C.

Results of chemical probing of bovine mt $tRNA^{Ser}_{AGY}$

Figure 1A–H shows the progressive denaturation of bovine mt $tRNA^{Ser}_{AGY}$ in high salt without Mg^{2+}. The set of lanes at 5°C is defined as representing the native structure in which all interactions are intact. The set of lanes at 90°C, shows the sequence of G, A, C and U residues in the tRNA in its completely denatured state when all residues are accessible to the modifying chemicals. These lanes are used as a reference for interpreting the pattern of reactivity in the sets representing other conformations. Although samples on each gel were equalized for radioactivity before electrophoresis, it is necessary to normalize band intensities between lanes to account for different amounts of radioactivity in different gels and, in each column, for slight differences in the extent to which a chemical reaction has been allowed to proceed. The normalized intensities are qualitatively expressed in Table I.

Band intensity reflects the availability of the corresponding base for chemical modification and, thus, involvement in hydrogen bonding or stacking. Therefore, one would expect the loss of an interaction to be expressed as the abrupt ap-

Fig. 1a

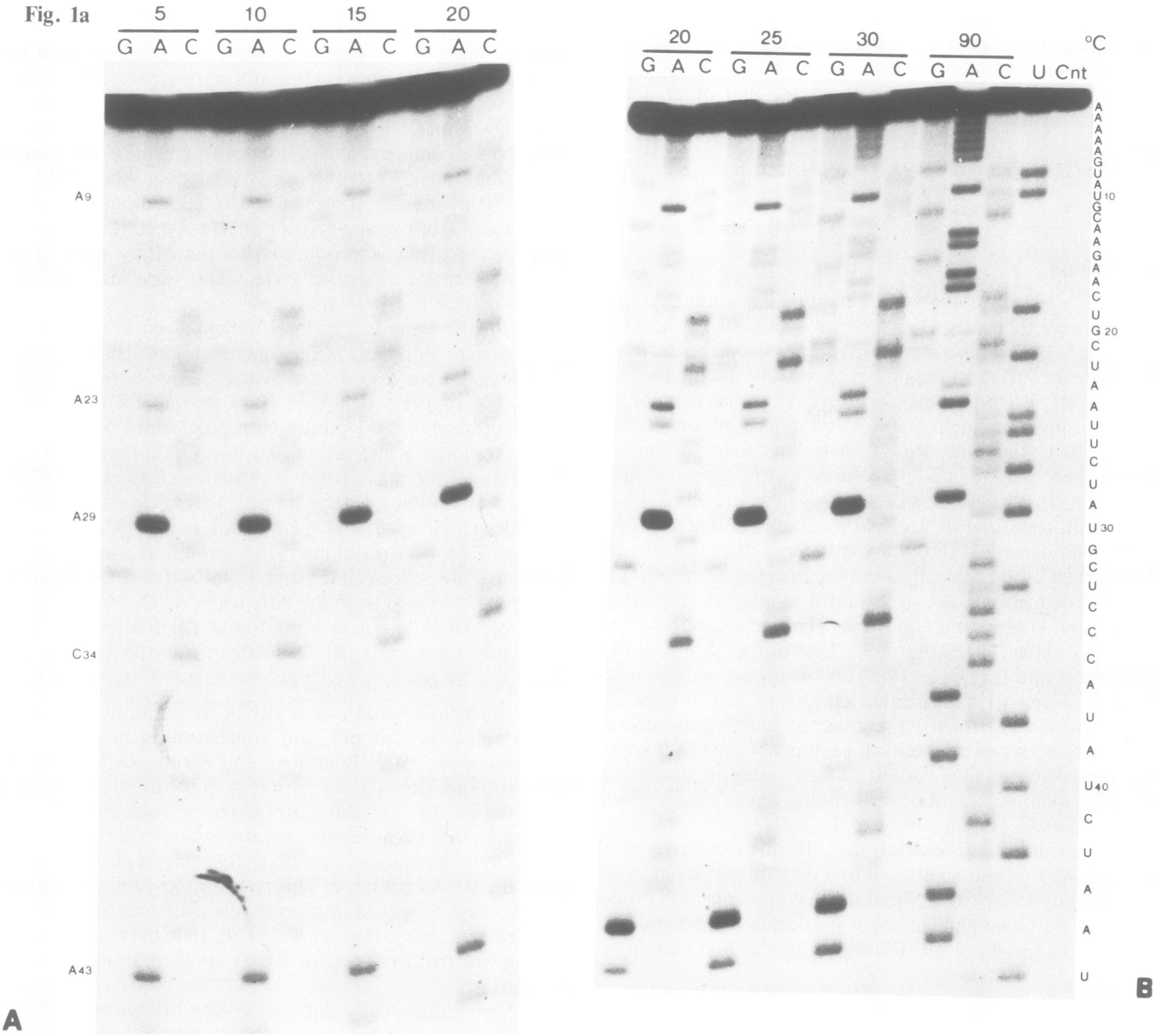


Fig. 1b

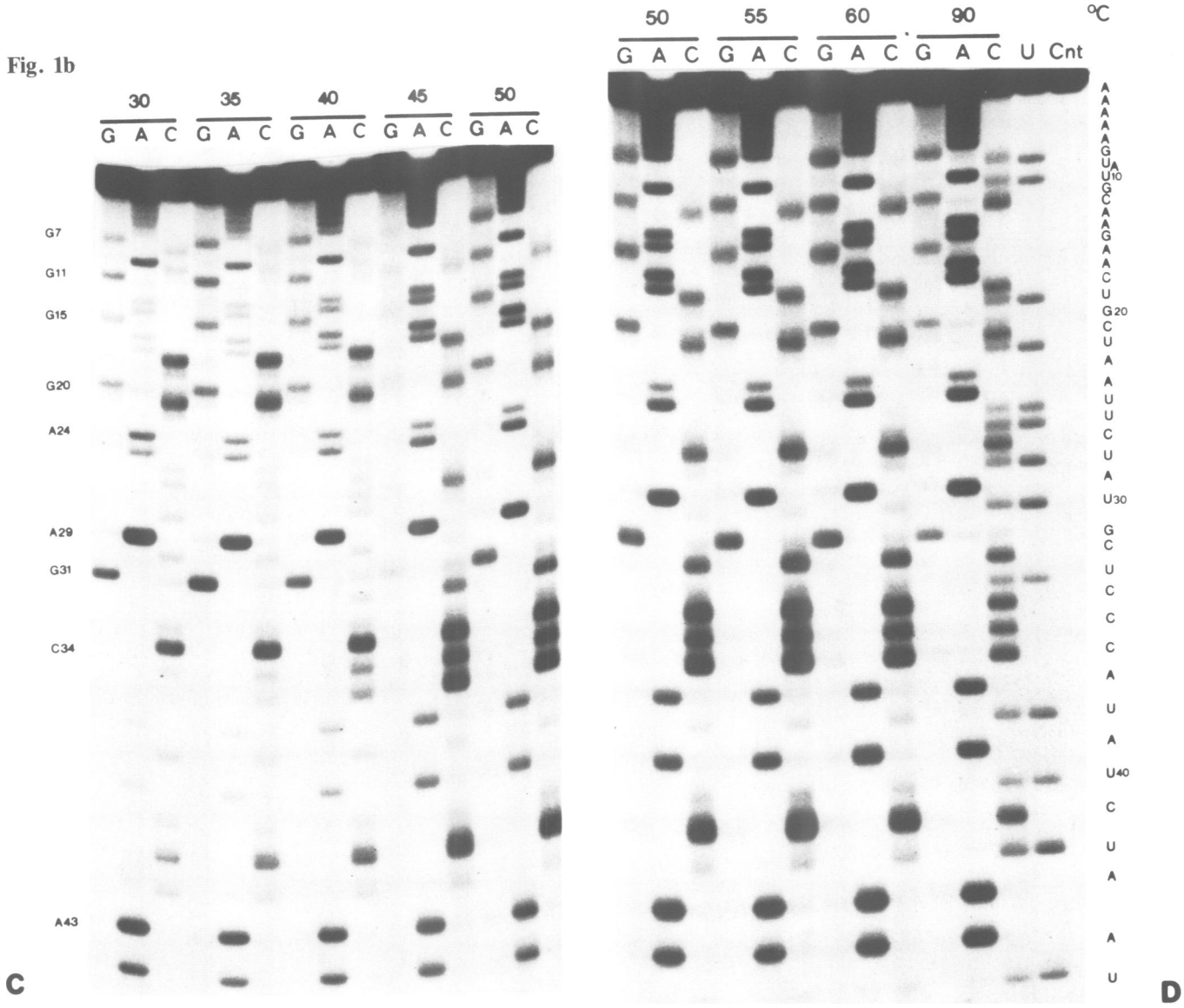


Fig. 1. Analysis by chemical probing of the structure of the bovine mt serine tRNA between 5° and 90°C. The band patterns directly locate and identify individual bases and their involvement in higher order structure. Samples of tRNA, prepared as described (Materials and methods), were subjected to the chemical modification reactions specific for guanosines (G), cytidines (C), adenosines (A) and uridines (U); the symbols in parentheses appear above each gel lane accordingly. The temperature at which the tRNA was probed is indicated above each set of corresponding gel lanes. Cnt (control), represents a RNA sample that has not been subjected to the chemical reactions. All chemical probing reactions were carried out in one experiment using the same RNA preparation and batches of chemicals. The nucleotides are numbered in the 5′ to 3′ direction. The 5′-terminal nucleotide has not been resolved. Weak bands corresponding to uridines appear in the C lanes due to the hydrazine step (Peattie, 1983) and do not reflect RNA conformation since the RNA is denatured at the pH of the hydrazine reaction. These bands can be identified using the U-specific lane in the 90°C set and will be disregarded. The heavy band at the top of each lane (**gels A – D**) shows that the modification reactions were limited in extent, strongly reducing the danger of base modifications altering the conformation of the tRNA. pCp (**gels F** and **H**), represents a sample of [5′-^{32}P]pCp; the 3′-terminal nucleotide of the tRNA (A63) aligns with the pCp marker band because strand scission by aniline is 3′ to the ribose ring of chemically modified A63. Samples on the same gel were equalized before loading except the 90°C samples on **gels B** and **F**, which were included as a sequence reference. Radioactivity of the samples was 1165 c.p.m. ± 7% (**gel A**), 1178 c.p.m. ± 8.5% (**gel B**), 1583 c.p.m. ± 7% (**gel C**), 1854 c.p.m. ± 12% (**gel D**), 1106 c.p.m. ± 7% (**gel E**), 1115 c.p.m. ± 7% (**gel F**), 1630 c.p.m. ± 9% (**gel G**) and 1817 c.p.m. ± 12% (**gel H**). Electrophoresis was at 1500 V for 7 h (**gels A – D**) or at 1900 V for 1 h before loading and for 1.5 h thereafter (**gels E – H**). Autoradiography was for 190 h (**gels A** and **B**), 170 h (**gels E** and **F**) or 140 h (**gels C, D, G** and **H**). The results of this analysis are summarized in Table I and Figure 2A – B.

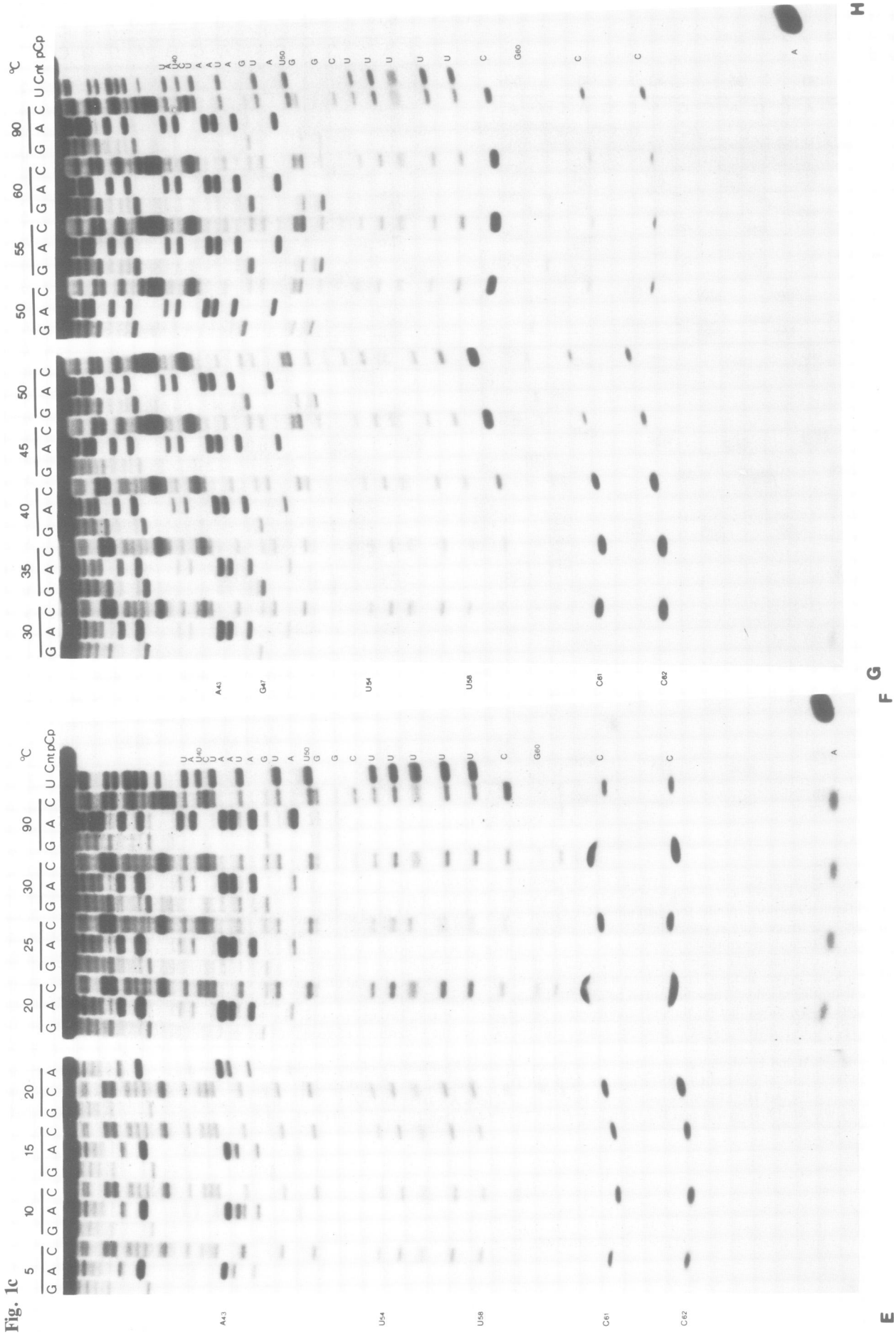
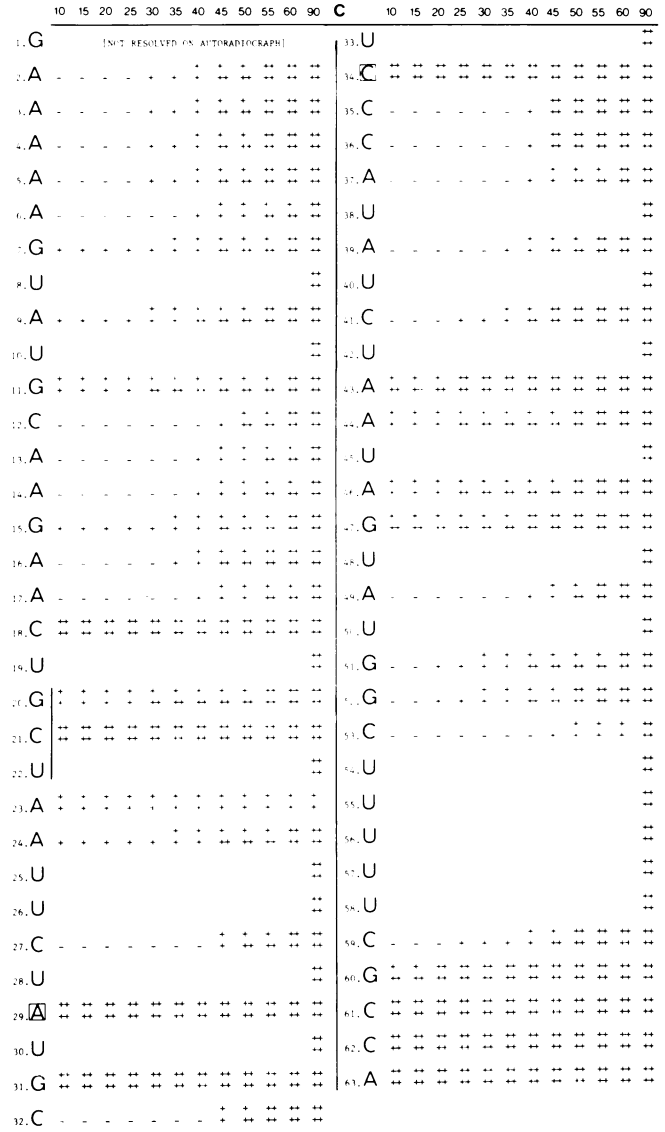


Fig. 1c

Table I. Normalized results of chemically probing the structure of the bovine mt serine tRNA between 10 and 90°C

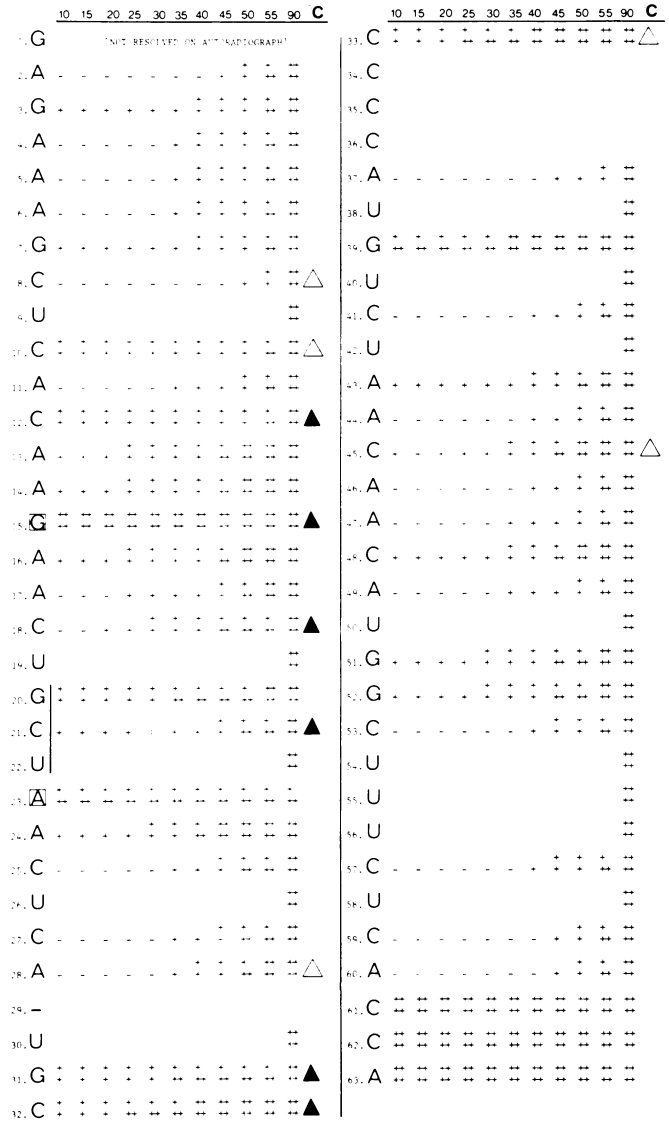


Relative intensities of bands in the autoradiographs (Figure 1) are qualitatively expressed on a five point scale from – (negligible intensity) to + + + + (full intensity). Corresponding temperatures are indicated along the top of the table. Corresponding nucleotide positions and the identity of bases in those positions are given in the two columns to the left of the 10°C column. Nucleotide No.1 (G1) is the 5′ end of the tRNA and the vertical bar marks the anticodon sequence.

Relative intensities in each row have been normalized to account for different amounts of radioactivity in different gels as inferred from duplicate samples and in each column to account for differences in the extent to which a chemical modification reaction was allowed to proceed. The latter adjustment was based on the assumption that A29 and C34 (shown boxed) were always fully exposed to the modifying chemical and, consequently, that the intensity of the corresponding bands should always be maximal, independent of temperature; the choice of C34 also functions as a standard in the G lanes since the initial modification reactions for G and C are identical (Peattie and Gilbert, 1980).

A23 is partially modified to t^6A (Arcari and Brownlee, 1980) and the corresponding band never reaches full intensity, even in the fully denatured state. It is assumed to have reduced sensitivity towards chemical modification and the strand scission reaction (c.f. Peattie and Gilbert, 1980; Peattie, 1982).

Table II. Normalized results of chemically probing the structure of the human mt serine tRNA between 10 and 90°C



Relative intensities of bands in the autoradiographs (not shown) are expressed as described in the legend to Table I. Nucleotide numbering is according to that of the bovine mt serine tRNA sequence. Additional information to that provided in Table I is marked with an open triangle at the corresponding positions. Corresponding positions in the human and bovine tRNAs for which substantially different probing results were obtained are indicated by solid triangles. A23 is presumed to be partially modified to t^6A and to have reduced sensitivity towards chemical modification and the strand scission reaction; therefore, the corresponding band never reaches full intensity. The autoradiographs contained no bands corresponding to C34, C35 and C36 but according to the DNA sequence of the gene all three positions should contain C (de Bruijn *et al.*, 1980). This is interpreted to mean that the three nucleotides are modified to m^5C since DMS is unreactive towards m^5C (Peattie and Gilbert, 1980; Peattie, 1982). This modification also occurs in the bovine tRNA and is characteristic for those positions in most tRNAs (Sprinzl and Gauss, 1982a, 1982b). All other details are as described in the legend to Table I.

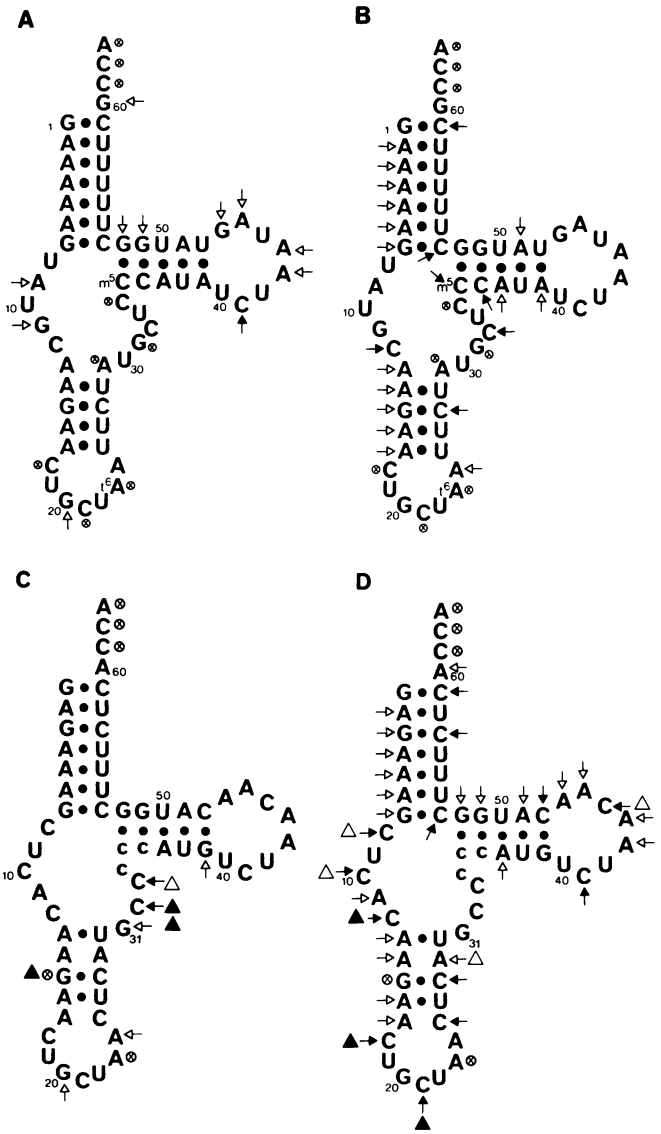


Fig. 2. Points of chemical modification in bovine and human mt $tRNA^{Ser}_{AGY}$. The diagrams are based on the results summarized in Tables I and II. The sequence of the two tRNAs are arranged in the predicted 'truncated cloverleaf' secondary structure. Nucleotides are numbered in the 5′ to 3′ direction. Black dots signify Watson-Crick base-pairing. **(A)** Chemical modifications in the bovine tRNA occurring between 5 and 40°C. ⊗ indicates that the corresponding base is never protected from the modifying chemical at any temperature. Arrows with open heads indicate that N-7 of the corresponding purines became modified at a temperature within the given temperature range but was protected below that temperature by participation in hydrogen bonding or by stacking of the purine. Arrows with solid heads signify the same for the corresponding cytidines. **(B)** Chemical modifications in the bovine tRNA occurring between 40 and 90°C. The arrows in **A** are not repeated. **(C)** Chemical modifications in the human tRNA occurring between 5 and 40°C. **(D)** Further chemical modifications in the human tRNA occurring between 40 and 90°C. Nucleotide numbering in the human tRNA is according to the bovine tRNA sequence **(A,B)** and the lower case letters at positions 34–36 signify that these nucleotides have not been determined or probed (see Table II). An open triangle signifies that the corresponding position in the bovine tRNA contains uridine, for which no structural probe is available and that the probing result for that position in human tRNA constitutes new information. A solid triangle signifies that the probing results at the corresponding positions in the bovine and the human tRNA are substantially different. The triangles used in this figure correspond to those in Table II. For reasons discussed in the text the highest temperature at which a band has a relative intensity of + + in Tables I and II has been chosen as that at which protection is lost and chemical modification occurs. For G_1 and the U residues no probing data are available.

pearance of a band of full intensity. However, many bands appear only gradually. This can be explained by the dynamic behaviour of RNA in solution, its structure being subject to transient 'breathing' changes. Thus, the pattern of reactivity at any given temperature does not reflect the conformation of a homogeneous population of molecules but rather the average conformation of a structurally heterogeneous population.

Transient changes are likely to be dependent on 'thermal motion' of the structure (Jack *et al.*, 1977; Holbrook *et al.*, 1978) and therefore will decrease with temperature. For this reason the native tRNA structure is represented by a set of probing data at 5°C, a temperature that bears no relation to that *in vivo*. Taking these considerations into account three general classes of bases are evident: (i) those that are not protected at any temperature from chemical modification; (ii) those that are protected from modification at 40°C or higher; and (iii) those that do not have total protection below 40°C. The latter two classes represent bases involved in interactions that are lost above and below 40°C, respectively. In Figure 2A and B bases in the 'truncated cloverleaf' secondary structure of bovine mt $tRNA^{Ser}_{AGY}$ have been classified accordingly. It is worth noting that the observed interactions are a persistent feature of the tRNA and do not change appreciably when probing is carried out in lower salt (50 mM sodium cacodylate, pH 7.5) or when EDTA is replaced by $MgCl_2$ (results not shown).

Chemical probing of human $tRNA^{Ser}_{AGY}$

From Figure 2A and B it is clear that several uridines occur in those loop regions of the bovine tRNA that can be expected to be involved in tertiary interactions (i.e., the loops except the anticodon loop). Unfortunately, the method used here does not provide a structural probe for uridines. The sequence of the bovine tRNA is only poorly conserved in its human counterparts (Figure 2C, D). However, four of the seven uridines occurring in the relevant loops of the bovine tRNA are replaced by cytidines in the human tRNA. Since cytidines can be chemically monitored, it was useful to duplicate the bovine $tRNA^{Ser}$ data by probing the human variant. The assumption is that both tRNAs will fold into a similar tertiary structure despite differences in nucleotide sequence and, therefore, that the human probing results can be used to complement the bovine data. In Table II the corresponding and normalized relative band intensities for the human tRNA are given. In Figure 2C and D bases in the 'truncated cloverleaf' structure of human $tRNA^{Ser}_{AGY}$ are classified according to whether they are never protected from chemical modification, are still protected at 40°C or higher or are not protected below 40°C (by analogy to the bovine tRNA). Again no changes in reactivity were detected when probing in the presence of less salt or of $MgCl_2$ instead of EDTA (results not shown).

Comparison of Tables I and II shows differences in melting behaviour between the bovine and human tRNA species. First, most bases in the human tRNA are protected from chemical modification up to a higher temperature than those in the bovine structure. Whether this difference is real or apparent remains unclear, since bovine and human probing data were obtained in separate experiments. Second, some bases have a markedly different pattern of reactivity towards the chemicals than the corresponding bases in the bovine tRNA (the most important ones are indicated in Table II and Figure 2C, D). The observed differences are not unexpected. The

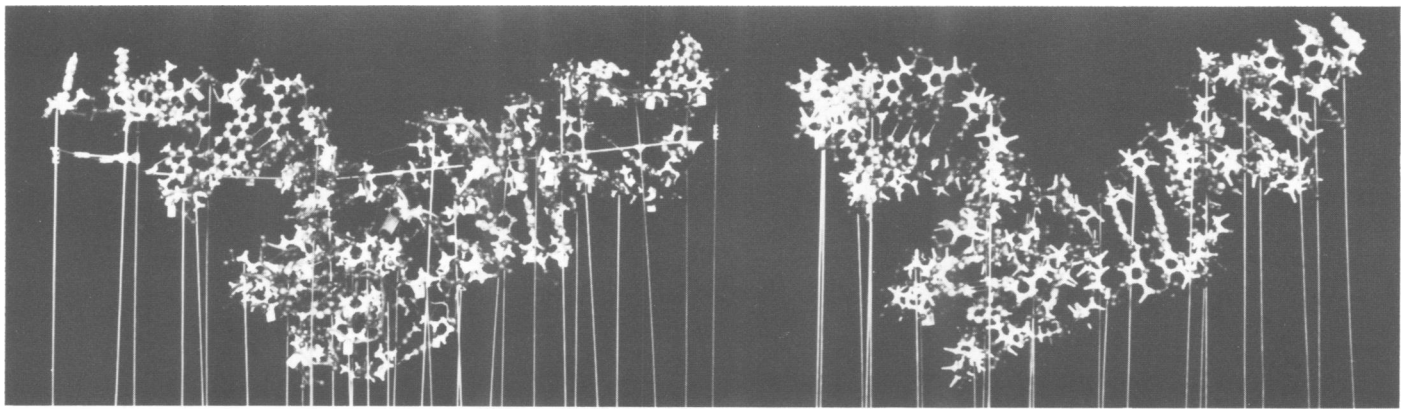


Fig. 3. Comparison of the proposed tertiary structure of bovine mt $tRNA^{Ser}_{AGY}$ with the established crystal structure of yeast $tRNA^{Phe}$ (Ladner *et al.*, 1975). Both tRNA structures are represented by 'Labquip' molecular models (1 cm per Å). The bovine tRNA is to the right.

human anticodon stem shows poor Watson-Crick base-pairing, the 'extra' or 'variable' loop is one nucleotide shorter than in the bovine tRNA and the nucleotide sequences of the TψC, variable and 'D-arm replacement' loops differ substantially.

Model building

The tertiary structure of yeast $tRNA^{Phe}$ is held together by several types of interaction: (i) hydrogen bonding between bases (in addition to those defined in the 'cloverleaf' secondary structure), (ii) hydrogen bonding between a base and the ribose-phosphate backbone, (iii) hydrogen bonding between two positions in the backbone, (iv) stacking of bases and (v) shielding interactions by water or other complexing molecules and ions (Ladner *et al.*, 1975; Quigley *et al.*, 1975; Jack *et al.*, 1976, 1977; Quigley and Rich, 1976; Hingerty *et al.*, 1978; Holbrook *et al.*, 1978). Many base pairs are not of the Watson-Crick type and some bases form triples rather than pairs.

Results of chemical probing are insufficient by themselves to establish a tertiary structure. They can give a detailed insight into which bases are involved in certain interactions, but other interactions are not monitored because the chemicals have no reactivity towards the atoms involved. In addition, no information is obtained as to the identity of the second member of an interacting base pair or regarding the topography of base-backbone, backbone-backbone and shielding interactions. Results of chemical probing have generally been in good agreement with tRNA crystal structure data derived from X-ray diffraction (Robertus *et al.*, 1974b; Rich and RajBhandary, 1976). They are therefore useful to test an existing tRNA structure or a preconceived structural model. We have built such a model of the bovine mt $tRNA^{Ser}_{AGY}$ structure using Labquip parts (Figure 3). In Figure 4A the proposed base-base interactions are indicated and in Figure 4B the tertiary structure is schematically represented by a tracing of the ribose-phosphate backbone. In the following sections the assumptions made in building the model are described and tested using the probing results. Consideration will be given to the applicability of the bovine model to other mammalian mt $tRNA^{Ser}_{AGY}$ species.

The 'truncated cloverleaf' secondary structure

From Figure 2B it is clear that the most stable interactions (i.e., those lost only above 40°C) mostly correspond to interactions in the secondary structure composing the 'truncated cloverleaf'. Protection of adenosines, and probably also guanosines, in the three stem regions can be interpreted as due to stacking and of cytidines due to Watson-Crick G.C base pairing. The 'truncated cloverleaf' secondary structure is to be expected from the nucleotide sequence by analogy to the full cloverleaf of other tRNAs. Conformation of this structure suggest that the loop regions in the 'truncated cloverleaf' of bovine $tRNA^{Ser}$ have a similar structural function to the TψC, D and extra loops in yeast $tRNA^{Phe}$ (Robertus *et al.*, 1974a).

Interpretation of the human data is less straight foward. The majority of interactions (Figure 2C, D) appear to melt in a one-step cooperative process. Nevertheless, the probing data are consistent with the secondary interactions defined in the 'truncated cloverleaf'. The major discrepancies between the human and bovine probing data concern identical bases and positions in the two tRNA structures (indicated by solid triangles in Figure 2C and D). They are confined to the lower half of the 'truncated cloverleaf' and could be related to the occurrence of the non-standard pairs A_{17}-C_{25} and A_{14}-A_{28} in the anticodon stem of the human tRNA. Both pairs are probably less stable that Watson-Crick base pairs and may introduce irregularities into the double-helical conformation of the stem which reduce the stabilizing effect of stacking (see below).

The proposed non-standard pairs A_{13}-A_{29} in the bovine tRNA and A_{14}-A_{28} in the human tRNA show distinctly different behaviour towards DEP. In the human tRNA both A_{14} and A_{28} appear to show a measure of stacking, but only A_{13} in the bovine pair shows this effect with A_{29} always available for chemical modification. The conclusion from this is that A_{13}-A_{29} is not a pair but the standard pair A_{13}-U_{30} is formed with A_{29} looped-out of the double-helical stem (Figure 4A) by rotation about its phosphodiester bonds without disturbing the helical continuity of its nearest neighbour base pairs (Lomant and Fresco, 1975). This reduces the bovine variable loop to four nucleotides as is the case in the human tRNA.

A-C and A-A pairs in tRNA double-helical stem regions

Analysis of published tRNA sequences (Sprinzl and Gauss, 1982a) shows that stems are overwhelmingly composed of standard Watson-Crick base pairs but that non-complementary pairs, such as G-U, A-C and A-A, occur with significant frequency. The most common pair, G-U, has been characterized in the yeast $tRNA^{Phe}$ crystal structure (Jack *et al.*,

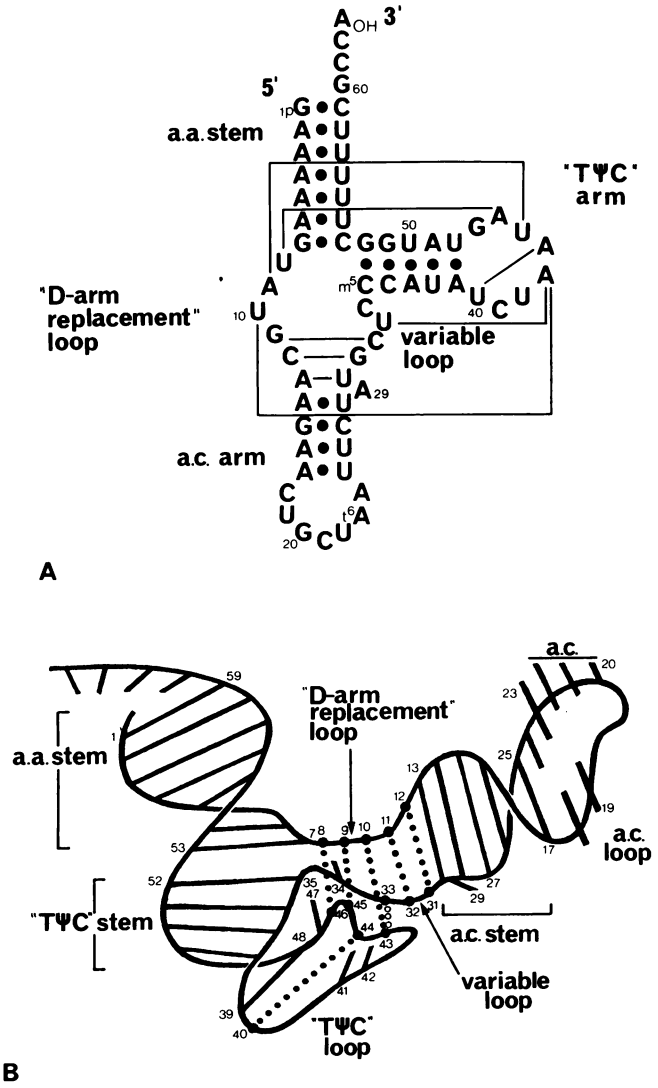


Fig. 4. Schematic diagram showing (**A**) the proposed 'truncated cloverleaf' formula and proposed tertiary connections and (**B**) the chain-folding and proposed tertiary interactions between bases in bovine mt serine (AGY) tRNA. Long straight lines indicate base pairs in the double-helical stems. Shorter lines represent unpaired bases. Dotted lines represent proposed base pairs in addition to those indicated in (**A**). The schematic drawing of the structure is based on the 'Labquip' model (Figure 3) which is viewed in a direction perpendicular to the plane through the three double-helical stems. Note that in **A**, as compared with Figure 2, the base A_{29} has been looped out.

1976) and denaturation studies demonstrated that G-U was relatively unstable promoting early melting of neighbouring A.U pairs (Rhodes, 1977).

Similarly, A-C and A-A pairs are found in stems (c.f. Figure 2). Indirect evidence for hydrogen-bonded A-C pairs has come from melting studies on long I.C double helices containing opposing A and C residues at low frequencies (Lomant and Fresco, 1975; Fresco *et al.*, 1980). It is therefore reasonable to assume that A-C and A-A pairs in tRNA stems use their intrinsic capacity for hydrogen bonding even though they are likely to be less stable than Watson-Crick pairs.

Figure 5 shows some potential hydrogen bonding schemes for these pairs. All four have anti-parallel glycosyl bonds as well as bond angles which are compatible with a double-helical configuration. The two A-C pairs are energetically unfavoured because they produce poor hydrogen bonding or require protonation. The fact that A-C pairs nevertheless occur may be explained if the 'cost' of forming them, expressed as an increase in free energy of the overall structure, is compensated, for example, by improved stacking. Which type of A-C base pair applies to A_{17}-C_{25} in human mt $tRNA^{Ser}_{AGY}$ is unclear, since the chemical probing data are consistent with both.

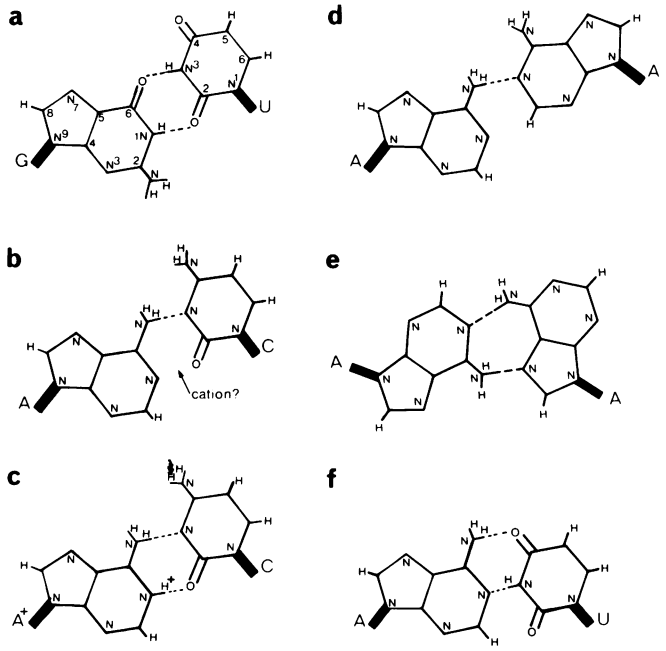


Fig. 5. Possible hydrogen bonding schemes for A-C or A-A base pairs in tRNA double-helical regions (Klug *et al.*, 1974; Topal and Fresco, 1976a, 1976b; and text). Only hydrogen bonding schemes have been considered that produce a geometry approximating that of a G-U wobble-pair (**a**) or a Watson-Crick base pair (**f**). (**b**) An A-C wobble-pair with one hydrogen bond; this combination leads to one unfulfilled hydrogen bond acceptor site (0–2) on C (Topal and Fresco, 1976b), unless 0–2 of C is shielded by a cation. (**c**) An A-C wobble-pair with two hydrogen bonds made possible by protonation of N-1 of the A member. (**d**) An A-A wobble-pair with one hydrogen bond. The distance between the two glycosidyl bonds is greater than in a Watson-Crick base pair, and would require considerable distortion of the double helix. (**e**) An A-A pair which would also require distortion of the backbones.

At least three hydrogen bonding schemes can be drawn for an A-A pair, two of which are shown in Figure 5. With one hydrogen bond (Figure 5d) the distance between the glycosyl bonds is probably too great to fit into a double helix. The scheme in Figure 5e would, however, fit in with some distortion of the backbone. The third scheme (not shown) is one in which there is no such distortion, but requires that one member of the pair undergoes a tautomeric shift to its imino form and the second takes up the *syn* conformation (Topal and Fresco, 1976a). The chemical probing data in human mt $tRNA^{Ser}_{AGY}$ are consistent with either of the last two possibilities: protection against chemical modification of A_{28} is lost at a significantly higher temperature (40°C) than in the case of A_{14} (25°C; Table II). This suggests that N-7 of A_{28} is protected by stacking while N-7 of A_{14} is protected by hydrogen bonding.

Comparison of homologous 'truncated cloverleaf' secondary structures

In addition to the nucleotide sequences of the bovine and human mt $tRNA^{Ser}_{AGY}$, RNA or DNA sequences for this tRNA have been reported from seven other mammalian

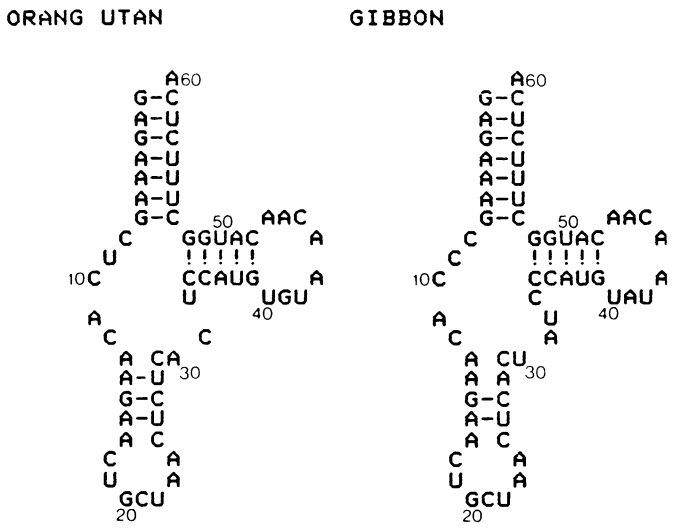


Fig. 6. The orang utan and gibbon mt $tRNA^{Ser}_{AGY}$ sequences arranged in predicted 'truncated cloverleaf' form. The tRNA sequences have been inferred from the sequence of their genes (Brown *et al.*, 1982). Post-transcriptional modifications have been omitted. Nucleotide numbering is according to that in the bovine tRNA sequence (Figure 2).

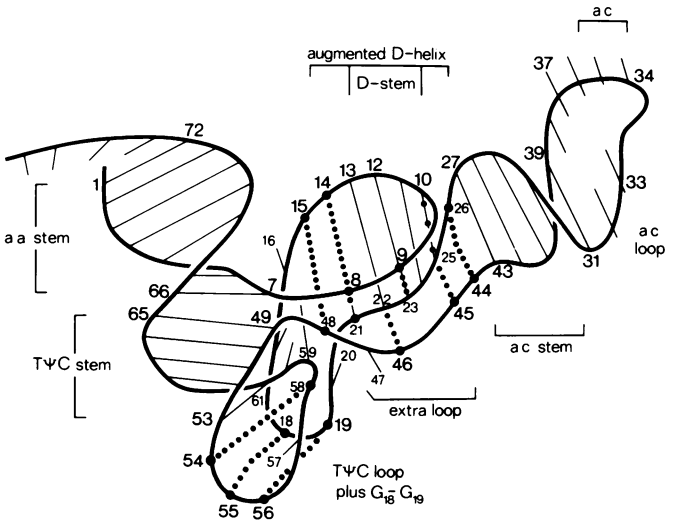


Fig. 7. Schematic diagram showing the chain folding and tertiary interactions between bases in yeast phenylalanine tRNA. Long straight lines indicate base pairs in the double-helical stems. Shorter lines represent unpaired bases. Dotted lines represent base pairs outside the helices. From Ladner *et al.* (1975).

species (hamster, Baer and Dubin, 1980; mouse, Bibb *et al.*, 1981; rat, Grosskopf and Feldmann, 1981; apes, Brown *et al.*, 1982). All can be folded in the 'truncated cloverleaf' secondary structure, but they vary considerably in sequence and also in the size of their 'TψC' and variable loops (c.f. Figure 6). Many coordinated base changes (Levitt, 1969; Klug *et al.*, 1974) are apparent in the stem regions, where purines and pyrimidines remain opposite each other and maintain base pairing. The comparison gives at least two additional sets of coordinated base changes suggesting that they represent conserved elements of tertiary structure. If A-C base pairing is permitted, seven out of the nine tRNAs are capable of extending the anticodon stem upwards by two base pairs (c.f. Figure 2C). The two exceptions are the bovine and gibbon tRNAs (Figures 2A and 6). As discussed previously there is reasonable evidence to assume that A_{29} in the bovine sequence is extra-helical and, in that case, similar extension of the anticodon stem is possible (Figure 4). This argument can be extended to the tRNA in gibbon. If A_{28} or C_{29} are looped-out of the double helix, not only can the anticodon stem be extended by two base pairs but overall base pairing in that stem would also be markedly improved.

The chemical probing data on the bovine tRNA (Table I; Figure 2A, B) clearly support the (Watson-Crick) base pairs $G_{11}.G_{32}$ and $C_{12}.G_{31}$ and suggest that these two pairs melt together with the secondary interactions. The probing results on the human tRNA are compatible with extension of the anticodon stem by the base pairs A_{11}-C_{32} and $C_{12}.G_{31}$ (Table II; Figure 2C, D) but less convincingly so. General instability of the human anticodon stem compared with the bovine stem probably results in different melting pathways for the two tRNAs.

Potential common elements in the tertiary structures of yeast $tRNA^{Phe}$ and bovine mt $tRNA^{Ser}_{AGY}$

The cloverleaf form of yeast $tRNA^{Ser}_{AGY}$ carries, in its general shape and in its sequence of bases, a partial blueprint of overall tertiary structure (Ladner *et al.*, 1975; Jack *et al.*, 1976; Kim, 1979; Figure 7). The structure is composed of a left and right arm, each ~60 Å long and at almost right angles to each other. The left arm consists of a long double helix in which the aminoacyl stem is stacked end-to-end on the TψC stem and on top of which the single-stranded CCA sequence stacks. The right arm is composed of the double-helical anticodon stem, on top of which the bases contained in the anticodon loop are stacked. The tips of the two arms are ~80 Å apart.

The thorax region connecting the two arms is composed of the D-stem, nucleotides 8 and 9, the extra loop and part of the D loop. The tertiary interactions between these four structural elements are arranged in such a way that they extend the continuous stack of bases in the D-helix to form the so-called 'augmented D-helix'. Tertiary interactions between invariant and semi-invariant bases in the TψC and D loops play a crucial role in maintaining the angle between the two arms. The TψC loop is anchored by stacking of the base pair T_{54}-m^1A_{58} on the end of the left arm. The anticodon stem makes an angle of ~20° with the augmented D-helix and therefore is not a simple continuation of it. Whereas the hydrogen bonding system is specific and gives the tRNA its characteristic shape, its stability is probably largely due to extensive stacking.

Since other bovine mt tRNAs display the complete cloverleaf secondary structure, their anticodon and CCA ends probably have a relationship like that in yeast $tRNA^{Phe}$. The three stem regions in bovine $tRNA^{Ser}_{AGY}$ (Figure 4) appear quite normal and it is reasonable to assume that they are arranged as in yeast $tRNA^{Phe}$. It is clear, however, that the thorax region must be different since the D arm is absent. There are three structural elements from which a thorax region could be built: the TψC loop, the variable loop and the D arm replacement loop.

It is important to note that all the nine known mammalian mt $tRNA^{Ser}_{AGY}$ sequences have either eight or nine (in rat), rather than seven, nucleotides in the TψC loop. One nucleotide extra in the TψC loop, if the backbone is locally stretch-

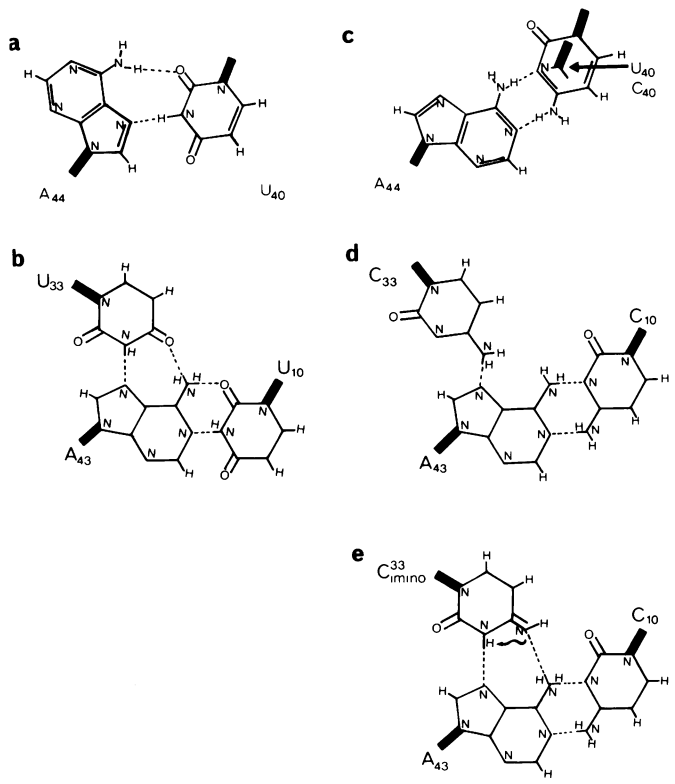


Fig. 8. Proposed non-Watson-Crick base pairs in the tertiary structures of bovine and human mt $tRNA^{Ser}_{AGY}$. **(a)** Reverse Hoogsteen-type base-pairing (Hoogsteen, 1963) is possible between U40 and A44 in the tRNAs from man, orang utan, gibbon, cow, hamster and mouse. The analogous base pair T54-m^1A58 has been confirmed in the crystal structure of yeast phenylalanine tRNA (Figure 7; Ladner *et al.*, 1975). **(b)** Alternative but equivalent base pairing between C40 and A44 in the tRNAs from chimpanzee, gorilla and rat. For comparison, the relative position of the glycosyl bond for U40 (see A) is shown when the glycosyl bond for A44 is held fixed. **(c)** Proposed hydrogen bonding in the base triple U10-A43-U33 in bovine mt $tRNA^{Ser}_{AGY}$, as suggested by the relative positions of those three bases in the 'Labquip' molecular model (Figure 3). **(d,e)** Possible equivalent base triple C10-A43-C33 in human mt $tRNA^{Ser}_{AGY}$ by analogy to the hydrogen bonding scheme shown in **c**. Scheme **(e)** would require C33 to take up its unfavoured imino tautomeric form involving one proton transfer.

ed, can increase the size of that loop by ~7 Å (equivalent to the length of a stack of three base pairs: c.f. distance between residues 8 and 9 in Figure 7). This gives the TψC loop the potential to interact with both the extra loop and the 'D-arm replacement' loop.

The TψC loop in yeast $tRNA^{Phe}$ contains one intra-loop base pair, T_{54}-m^1A_{58}, which is stacked on the end of the TψC stem. This base pair is of the 'reverse Hoogsteen' type (Hoogsteen, 1963; Ladner *et al.*, 1975; c.f. Figure 8a). Examination of the known mt $tRNA^{Ser}_{AGY}$ sequences shows that six of the nine tRNAs could make the equivalent reverse Hoogsteen base pair U_{40}-A_{44}, dividing the enlarged loop into two domains and so restricting its interactive capabilities. In chimpanzee, gorilla and rat the corresponding pair is C_{40}-A_{44}. C and A cannot form a stable 'reverse Hoogsteen' pair because N-3 in C_{40} is not protonated. However, an A-C pair with parallel glycosyl bonds (Figure 8c) is an excellent alternative for a reverse Hoogsteen A-U pair. If the two pairs are overlapped and aligned with respect to the glycosyl bond of A_{44}, then the glycosyl bonds of U_{40} and C_{40} have almost the same angle to the backbone. The shift between U_{40} and C_{40} is no greater than that produced by a wobble base and can easily be accommodated.

For both the bovine and human $tRNA^{Ser}_{AGY}$ species, the probing data are consistent with the proposed reverse-Hoogsteen pair U_{40}-A_{44}. A_{44} is protected from chemical modification to a significantly higher temperature than A_{43} (Tables I and II). A reasonable explanation for this is that both A_{43} and A_{44} are stacked but that, in addition, N-7 of A_{44} is involved in hydrogen bonding.

The tertiary structure model for bovine mt $tRNA^{Ser}_{AGY}$

Above, several arguments have been put forward regarding the spatial folding of bovine mt $tRNA^{Ser}_{AGY}$ and the base pairs that define this structure. Each of these arguments is plausible by itself and supported by the available chemical probing data. The molecular model (Figures 3 and 4) shows that they are also compatible with each other.

The left and right arms of the structure have approximately the same angle between them as in yeast $tRNA^{Phe}$. The two arms of the molecule are connected by the anti-parallel chains of the variable and 'D-arm replacement' loops. The anticodon arm has been extended with the Watson-Crick base pairs G_{11}.C_{32} and C_{12}.G_{31} as predicted from the 'truncated cloverleaf' comparisons. The TψC loop has been divided into two domains by the reverse-Hoogsteen base pair U_{40}-A_{44} which, by analogy to T_{54}-m^1A_{58} in yeast $tRNA^{Phe}$, stacks on the end of the TψC stem.

Model building itself suggested three additional tertiary base-base interactions. Two of these, U_8-A_{46} and A_9-U_{45}, are of the standard Watson-Crick type. The third interaction concerns the base triple U_{10}-A_{43}-U_{33} (Figure 8b). Hydrogen bonding between U_{10} and A_{43} is of the 'reverse Watson-Crick' type in which U has rotated 180° around its N-3 hydrogen bond. Hydrogen bonding between U_{33} and A_{43} is according to Hoogsteen (1963). Both U_8.A_{46} and A_9.U_{45} are supported by the bovine probing data in that A_9 and A_{46} appear to be stacked (Table I). The probes are inadequate for monitoring the base triple since two of the bases involved are U residues. A_{43} shows little protection from the chemical probe. Either N-7 of A_{43} is not involved in the interaction, in which case the suggested base triple is not made, or the base triple, as a whole or in part, melts very easily.

Four residues in the thorax region (nos. 34, 41, 42 and 47) have no apparent structural function in the bovine model (Figure 4B). The probing data (Table I) suggest that at least some of these are protected from chemical modification by hydrogen bonding. This could be the case if they are involved in base-backbone interactions as found in yeast $tRNA^{Phe}$ (Jack *et al.*, 1976; Hingerty *et al.*, 1978).

The thorax region of mt $tRNA^{Ser}_{AGY}$ in other animals

It is not clear whether the three tertiary hydrogen bonding schemes suggested by model building are equally applicable to other mt $tRNA^{Ser}_{AGY}$ structures. With the exception of A_{46} and A_{43} the involved bases are not conserved and no coordinate base changes are apparent. The proposed base pair U_8-A_{46} is conserved in the hamster and rat tRNAs only and is replaced by A_8-A_{46} in mouse or by C_8-A_{46} in the remaining serine tRNAs. The combination C_8-A_{46} in human mt $tRNA^{Ser}_{AGY}$ would be consistent with the probing results, according to which N-3 of C_8 is protected and A_{46} appears to be stacked (Table II).

The proposed base pair A_9.U_{45} is conserved in hamster and, by a coordinated base change, in mouse. In the remaining tRNAs the equivalent base pair would involve two

pyrimidines or, in the case of rat mt $tRNA^{Ser}_{AGY}$, two purines. If possible physically, they would hardly contribute to stacking in the thorax region and therefore destabilize that region unless the neighbouring base pairs are of a different type too.

The proposed base triple U_{10}-A_{43}-U_{33} (Figure 8c) is conserved in hamster and mouse. Since the gibbon and orang utan tRNAs effectively have a variable loop of only three nucleotides, it is probably not possible to introduce the equivalent base triple without 'overstretching' the variable loop between residues 33 and 35 (c.f. Figure 4B). In the human tRNA the equivalent base triple would be C_{10}-A_{43}-C_{33}. The connection C_{10}-A_{43} can easily be made (Figure 8b). The connection A_{43}-C_{33} would involve only one hydrogen bond or require C_{33} to shift to its imino tautomeric form (Figure 8d, e). This is equally true for the tRNAs from chimpanzee (C_{33}) and rat (A_{33}). Only in the bovine, hamster and mouse tRNAs do all interactions appear to be comparable. The other serine tRNAs probably use different base pairing arrangements in this part of the thorax region.

Dimensions of the bovine mt $tRNA^{Ser}_{AGY}$ structure

The yeast $tRNA^{Phe}$ and our model for bovine mt $tRNA^{Ser}_{AGY}$ (Figures 4 and 7) have one major difference. Whereas the thorax region of yeast $tRNA^{Phe}$ is composed of an array of seven stacked base pairs or triples (the 'augmented D-helix'), the bovine thorax region only has five such stacks. From the constant five-nucleotide size of the D-arm replacement loop and from the backbone topology in the tRNA model it is evident that the number of stacks could possibly be increased to six through stacking of unpaired bases (e.g., from residue 47) or additional base-backbone interactions, but that a stack of seven base pairs or triples is very unlikely. Only one of the mt tRNAs, $tRNA^{Leu}_{UUR}$, has retained most of the 'universal' features of non-mitochondrial tRNAs (Anderson *et al.*, 1981, 1982a, 1982b) and would be capable of forming a tertiary structure like that of yeast $tRNA^{Phe}$, but it is possible that this may have a function other than in protein synthesis.

The observation that bovine $tRNA^{Ser}_{AGY}$ is smaller than yeast $tRNA^{Phe}$ has some interesting implications. If the assumption that all bovine mt tRNAs have the same dimensions in order to be able to interact with the same mitochondrial ribosome is correct, then their structures should be correspondingly smaller too. Many mammalian mt tRNAs have D or TψC loops of only three or four nucleotides (Anderson *et al.*, 1982b), whereas in non-mitochondrial tRNAs these loop sequences have at least seven nucleotides. It is therefore doubtful whether mt tRNAs can fold in the exact image of yeast $tRNA^{Phe}$. If mammalian mt tRNA structures were slightly smaller, this problem need not arise. At least three mammalian mt tRNAs, $tRNA^{Ser}_{AGY}$ from orang utan and gibbon (the latter in its revised form; Figure 6) and bovine $tRNA^{Leu}_{CUN}$ (Anderson *et al.*, 1982b), have only three nucleotides in the extra loop. In a yeast $tRNA^{Phe}$-type structure a three nucleotide extra loop is physically impossible, since it would require the ribose-phosphate backbone to stretch beyond its capacity (Clark and Klug, 1975). If the thorax region of these tRNAs were shorter by only one stacked base pair (~3.5 Å), then a three nucleotide variable loop could be accommodated.

Concluding remarks

An attempt has been made to determine the tertiary structure of an unusual tRNA on the basis of sequence comparisons, chemical probing and the crystal structure of yeast $tRNA^{Phe}$. This approach is rapid but not as definitive as X-ray analysis. On the other hand, chemical probes are very sensitive. This is illustrated in Figure 1 by the band corresponding to A_6. Except when the tRNA is completely denatured this band is consistently weaker than those corresponding to A_5, A_4 and A_3. The reason for this is clear from the bovine mt $tRNA^{Ser}_{AGY}$ tertiary structure (Figure 4), which shows that the base pair A_6.U_{54} is not only adjacent to the 'strong' pair G_7.C_{53} but in close proximity to two additional G.C pairs that stabilize the gap between residues 7 and 35 in the long double-helix of aminoacyl and TψC stems. One can confidently predict that this arrangement confers enhanced stability to this particular region of the structure. The logical consequence is (c.f. Rhodes, 1977) that, upon denaturation, the pair A_6.U_{54} will be prevented from melting along with the equally weak neighbouring A.U pairs.

It is remarkable that bovine mt $tRNA^{Ser}_{AGY}$, despite having an anomalous secondary structure and using a different set of interactions, is capable of folding into a tertiary structure that is similar to that of yeast $tRNA^{Phe}$ (Figure 3). The absent D loop is partly compensated for by an extra nucleotide in the TψC loop which comes to rest in the thorax region, where it interacts with three residues in the D-arm replacement loop. The remaining two residues in the D-arm replacement loop form base pairs with the variable loop. In this way all five bases in the D-arm replacement loop are involved in base pairs which stack on the anticodon stem to replace the augmented D-helix in the thorax of the molecule.

The bovine mt $tRNA^{Ser}_{AGY}$ structural model is no more than a reasonable prediction. Its value lies not in accuracy of detail but in satisfactorily combining a variety of data from different sources in a structure that makes sense in terms of design and stability. Compared with other tRNA structures it may appear weak in having more unusual and energetically less favourable base pairs and, possibly, in lacking strong metal binding sites. However, it should derive a considerable degree of stability from extensive stacking throughout the molecule, and it only needs to recognize a very limited number of codons (55 AGY codons against 221 UCN codons; Anderson *et al.*, 1982a).

Materials and methods

Fresh bovine hearts were from a local slaughterhouse. Human placentas were obtained at term from normal or caesarean section deliveries.

Polynucleotide kinase and T4 RNA ligase were purchased from P.L. Biochemicals, Inc., Milwaukee, WI and [γ-^{32}P]ATP (~5000 Ci/mmol) from Amersham International plc, UK. DEP was purchased from B.D.H. Ltd., Poole, UK, and DMS and hydrazine (95% minimum by titration) from Eastman Kodak Company, Rochester, NY. RNA models were built using Labquip Nicholson Molecular Models (Labquip, Reading, UK). All polyacrylamide gels were prepared from a stock solution containing 38% acrylamide and 2% N,N'-methylenebis-acrylamide.

Isolation of mitochondrial nucleic acids

Mitochondria were isolated from bovine hearts (average weight after removal of fat and connective tissue: 700 g) as described (Smith, 1968), except that mitochondria were washed 3–4 times. Mitochondria from human placentas were also isolated in this way but with the following modifications. After removing membrane and umbilical cord the remaining tissue (average weight ~350 g) was diced and washed three times in isotonic saline. Homogenization in the Waring blender was for 30 s at medium speed. After two washing cycles the mitochondria were resuspended in buffer B supplemented with $MgCl_2$ (2 mM final concentration) and DNase I (final concentration of 25 μg/ml). DNase I treatment was for 30 min at room temperature. The suspension was then chilled and subjected to two more washing cycles. The mitochondrial pellets were resuspended in 75 mM NaCl, 5 mM EDTA and

Table III. Incubation times needed to obtain a comparable level of base modification by DMS or DEP at various temperatures

Temperature	Incubation time (min)		Temperature	Incubation time (min)	
	DMS	DEP		DMS	DEP
5	115	240			
10	85	185	40	17	23
15	58	150	45	14	14
20	41	120	50	12	25[a]
25	30	80	55	10	15[a]
30	25	50	60	8	10[a]
35	20	36	90	1	15[b]

At each given temperature a suitable incubation time of RNA with the modifying chemical was determined by trial and error. The aim was to obtain a comparable level of modification (as expressed by band intensity on the autoradiographs) of those bases in the tRNA structure that were obviously completely exposed to the chemical at all temperatures (c.f. A29). The intensity of the band pattern at 90°C was used as a standard. [a]50% and [b]75% less DEP added.

25 mM Tris-HCl, pH 7.5 and lysed at room temperature by addition of SDS to an end concentration of 2%. The lysate was twice extracted with an equal volume of buffer-saturated phenol and a third time using 50% (v/v) buffer-saturated phenol and 50% chloroform/amylalcohol (24:1). Nucleic acid was precipitated overnight with 2.5 volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.5) at −20°C.

Isolation of mitochondrial tRNA$^{Ser}_{AGY}$

The mitochondrial nucleic acid fraction was dissolved in 0.25 M NaCl, 0.1 M Tris-HCl, pH 7.5 and applied to a O-diethylaminoethyl cellulose column (column volume 12 ml in a 10 ml disposable pipette; Holley *et al.*, 1961). RNA was eluted in 1 M NaCl, 0.1 M Tris-HCl, pH 7.5 and precipitated with ethanol as described. The RNA was dissolved in 0.2 ml 0.01 M EDTA, 0.01 M Tris-HCl, pH 8.0 and added to an equal volume of 98% formamide. This mixture was incubated at 50°C for 5 min, rapidly cooled on ice and applied to a 10% polyacrylamide-7 M urea gel (0.1 x 20 x 40 cm) in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Electrophoresis was at 700 V for 4 h. The RNA was visualized as described (Hassur and Whitlock, 1974) and the band containing tRNA$^{Ser}_{AGY}$, recognizable because of its position ahead of the bulk of tRNA, excised from the gel and sliced. The tRNA was eluted overnight and at room temperature in 50% formamide, 0.5% SDS, 0.5 M ammonium acetate and 1 mM EDTA (Peattie, 1983). The average yield from one bovine heart was ~10 μg and from one human placenta ~4 μg of tRNA$^{Ser}_{AGY}$.The RNA was ethanol precipitated and dissolved in water at 0.4 mg/ml.

Preparation of labelled tRNA

Preparation of [5′-^{32}P]cytidine 3′,5′-biphosphate and labelling of the tRNAs (~0.4 μg per experiment) were as described (England and Uhlenbeck, 1978; Bruce and Uhlenbeck, 1978). The mixture was then combined with 0.5 vol of 98% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue, heated at 50°C for 3 min and immediately applied to a 12% polyacrylamide-7 M urea gel (0.035 x 20 x 40 cm) in TBE buffer. After electrophoresis at 1500 V for 2 h the gel was dismounted, covered with 'Saranwrap' cling film and exposed to Fuji RX film. Exposures of 0.5 – 1 min were generally sufficient to locate the RNA. The RNA bands were excised from the gel and eluted as described previously, but at 37°C for 5 h. The eluted RNA was ethanol precipitated overnight at −20°C without addition of carrier and collected by centrifugation in a Beckman SW 60 rotor at 55 000 r.p.m. and 0°C for 60 min. The precipitate was washed with cold ethanol, recentrifuged, dried and finally dissolved in 250 μl of autoclaved, deionized water. A typical yield in terms of radioactivity, as determined by Cerenkov counting, was 1.3 x 10^6 c.p.m.

Renaturation and chemical probing

For chemical probing between 0 and 60°C three tRNA samples for each chosen temperature were prepared. About 10 μl of the tRNA solution described above was transferred to an Eppendorf tube and mixed with 0.2 ml of 50 mM sodium cacodylate-HCl, pH 7.0, 120 mM NaCl and either 1 mM EDTA or 1 mM $MgCl_2$. Samples for probing at 90°C always contained EDTA and, if to be treated with DMS, were mixed with 0.3 ml instead of 0.2 ml of the salt solution. The samples were incubated at 37°C for 10 min and then gradually chilled to 5°C. Probing was essentially carried out as described (Peattie, 1979, 1982; Peattie and Gilbert, 1980) but over a wide temperature range and with the indicated adjustments to reaction time and the amount of added DEP (Table III).

Electrophoresis and autoradiography after probing

Samples were prepared *in duplo* and preferably contained 10%, but never more than 25%, of the RNA in the original samples in order to avoid subsequent distortion of band patterns due to a high salt concentration. The samples were equalized by Cerenkov counting (± 10%) and evaporated to dryness on a vacuum line. Each sample was dissolved in 3 μl of 8 M urea, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.05% xylene and cyanol and 0.05% bromophenol blue. They were heated at 90°C for 30 s and immediately applied to two 20% polyacrylamide-7 M urea gels (0.035 x 20 x 40 cm) in TBE buffer. Control samples of unmodified RNA were also applied to both gels and a sample of [5′-^{32}P]pCp to one of the gels. Electrophoresis of the latter was at 1900 V for 1 h before application of the samples and ~1.5 h thereafter until the bromphenol blue marker had migrated 15 cm. Electrophoresis of the other gel was at 1500 V for 7 h until the xylene cyanol marker was 7 cm from the bottom edge of the gel. After electrophoresis the gels were dismounted, covered with 'Saranwrap' cling film and exposed to presensitized Fuji RX film at −20°C in the presence of an intensifying screen (Laskey, 1980).

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