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Substrate recognition by human RNase P: identification of small, model substrates for the enzyme

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RNase P from HeLa cells can efficiently cleave tRNA precursor molecules *in vitro* but cannot cleave potential substrates from which the D, anticodon and variable loops and stems of the tRNA moiety have all been removed. However, molecules from which the latter subdomains have been removed individually do serve as substrates. We show here that molecules that contain only a 5′ leader sequence, the acceptor stem and the T stem and loop of the tRNA domain, and a bulge as small as one nucleotide downstream from nucleotide 7 in the tRNA sequence at the junction of the two stems, can serve as substrates for human RNase P. The identity of the nucleotide in the bulge is important in determining both the efficiency of the cleavage and the conformation of the substrate and/or the enzyme–substrate complex. We also show that the human enzyme locates the appropriate site for cleavage of its substrates in part by 'measuring' the length of the helices in the acceptor and T stems in both model and natural substrates.

Key words: bulged nucleotide/human RNase P/measuring RNA/model substrates

Introduction

RNase P is the endoribonuclease that cleaves precursor tRNA (ptRNA) transcripts to generate the 5′ termini of mature tRNAs. This essential enzymatic activity has been found in all cells examined, both prokaryotic and eukaryotic (Altman *et al.*, 1988). RNase P from the eubacterium *Escherichia coli* has been characterized in great detail: the enzyme is composed of a protein with a molecular mass of ~13.7 kDa (C5 protein) and an RNA of 377 nucleotides (M1 RNA). In the presence of a high concentration of Mg^{2+} ions, M1 RNA can catalyze the cleavage reaction by itself *in vitro*. However, the addition of C5 protein, which allows formation of the holoenzyme (RNA plus protein), dramatically increases the rate of cleavage (Guerrier-Takada *et al.*, 1983; Guerrier-Takada and Altman, 1984).

Human RNase P is also a ribonucleoprotein (Bartkiewicz *et al.*, 1989). It consists of one RNA component (H1 RNA) and one or more proteins. The similarity between the proposed secondary structures of M1 RNA and H1 RNA indicates that they may share a common ancestor (Bartkiewicz *et al.*, 1989; Forster and Altman, 1990). However, RNase P from human cells is different from the enzyme from *E.coli* in at least two respects: (i) the RNA subunit of RNase P from *E.coli* is catalytically active alone *in vitro*, while the human RNase P is active only as a ribonucleoprotein complex; and (ii) the substrate specificity of human RNase P seems much narrower than that of the enzyme from *E.coli*. For example, the RNase P holoenzyme from *E.coli* and its catalytic RNA subunit, in the absence of protein, each cleave not only normal tRNA precursors but also a model substrate that contains only a 5′ leader sequence, the acceptor stem and the T stem–loop of $tRNA^{Phe}$ from *E.coli* (designated pAT-1; McClain *et al.*, 1987). However, pAT-1 is not a substrate for the human enzyme. RNase P from human cells appears to be less promiscuous than the enzyme from *E.coli* in the sense that model substrates must retain more features of the structure of a tRNA precursor if they are to function as efficient substrates for the enzyme (Yuan *et al.*, 1992). In this report we identify the minimal structural features of a precursor to a conventional tRNA that are recognized by human RNase P and we describe a small, simple, model substrate for this enzyme.

Results

Cleavage by human RNase P of precursor tRNAs that contain large deletions

Human RNase P cannot cleave pAT-1 (Yuan *et al.*, 1992); neither can it cleave an analogous substrate, known as pATy (see below), that is derived from the precursor to $tRNA^{Tyr}$ from *E.coli* (pTyr; Figure 1A). Several new derivatives of pTyr that contain deletions of different domains, as shown in Figure 1B–H, were synthesized by transcription *in vitro* from appropriate DNA constructs (see Materials and methods) to allow us to explore systematically those parts of the tRNA domain of the ptRNA, in addition to the acceptor and T stem, that are needed for recognition by the human enzyme. Although removal of the D stem and loop from pTyr led to a decrease in the ability of this derivative (ΔD) to serve as a substrate, ΔD could still be cleaved at a considerable rate (Figure 2, lanes 3 and 4). ΔAC, a mutant in which the anticodon stem and loop had been completely deleted, proved to be the most efficient substrate among those tested. ΔAC was cleaved at a rate similar to that of the parent molecule pTyr (Figure 2, lanes 1, 2, 5 and 6). Reduction in the size of the variable loop to two nucleotides (ΔV-2) did not markedly affect susceptibility to cleavage by RNase P. Note that ΔV-2 had only one additional nucleotide in the variable loop, as compared with ΔV-1, but its ability to serve as a substrate was considerably greater (Figure 2, lanes 7–10), an indication that an important conformational feature of the substrate was determined by a variable loop of a minimum size. Although the anticodon domain and most of the variable loop were

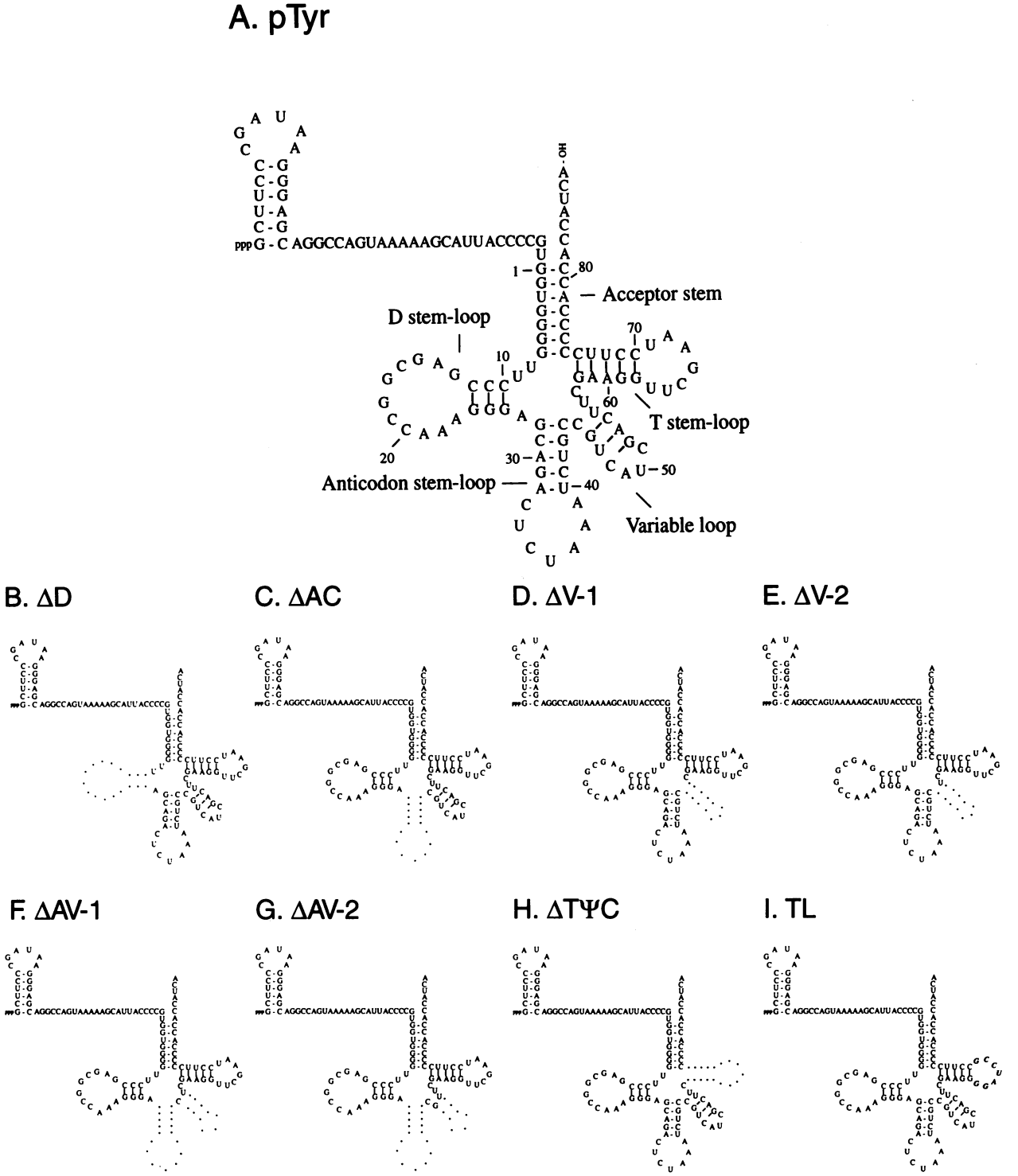

Fig. 1. Sequences and secondary structures of (**A**) the precursor to $tRNA^{Tyr}$ su3 (pTyr) and (**B**–**I**) its derivatives.

separately dispensable, the combination of such deletions (ΔAC and ΔV-2) led to a substrate (ΔAV-1) with much reduced susceptibility (Figure 2, lanes 11 and 12) to cleavage by human RNase P. ΔTΨC was not cleaved at a detectable rate under our conditions (Figure 2, lanes 15 and 16). One additional derivative, designated TL and shown in Figure 1I, was constructed in which the sequence in the T loop was altered (compare with Figure 1A) such that some of the hydrogen bonds responsible for maintaining the tertiary structure of the RNA were eliminated. This alteration of the T loop also eliminated the ability of TL to be cleaved by human RNase P (Figure 2, lanes 17 and 18). These results show that human RNase P can effectively cleave a ptRNA substrate from which any of the D, anticodon or variable stem–loops have been removed, while the T loop and some additional feature of the T stem and acceptor stem are important for efficient cleavage.

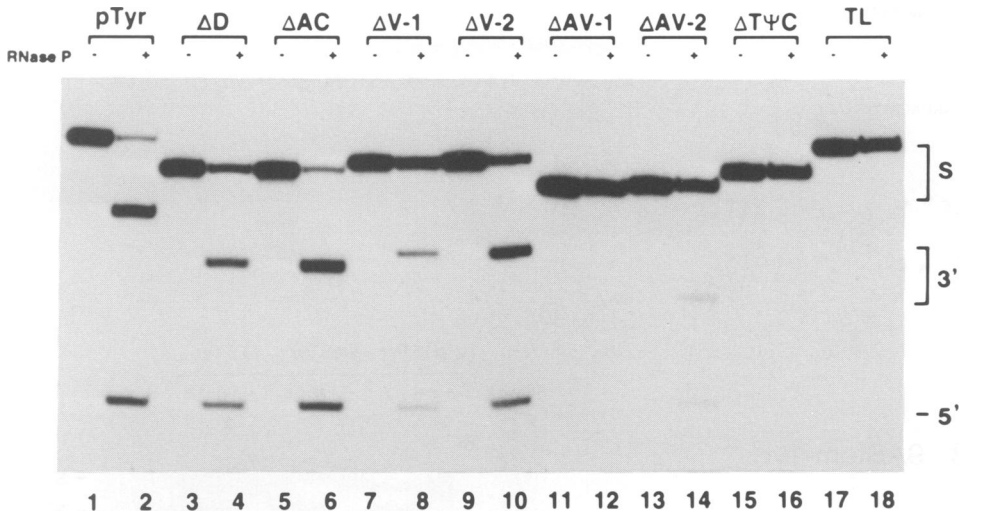

Fig. 2. Assay of cleavage of pTyr and the mutant derivatives by human RNase P. pTyr and the mutant derivatives, as indicated above each lane, were incubated with (even-numbered lanes) or without (odd-numbered lanes) human RNase P for 30 min under the conditions described in Materials and methods. The cleavage products were analyzed in an 8% polyacrylamide−7 M urea gel. 'S' indicates substrate, '5'' the product of cleavage by RNase P that contains the 5′ leader sequence, and '3'' the product of cleavage that contains the 3′ proximal sequence. The brackets indicate that not all the S and 5′ species had the same molecular mass.

Kinetic analysis of model substrates

A kinetic analysis was performed of the reactions of RNase P with three of the mutant substrates, ΔD, ΔAC and ΔAV-2, to determine the Michaelis constants (K_m) and maximum velocities (V_{max}). The kinetic parameters obtained from the reactions with these mutants are listed in Table I and can be compared with those obtained with the parent substrate pTyr. Removal of the anticodon domain from the substrate resulted in a 4-fold increase in K_m (40 versus 10 nM). Combined deletion of the anticodon and most of the variable loop (ΔAV-2) brought about a further increase in the K_m (to 110 nM). As described below, when all three non-essential domains (the D stem−loop, the anticodon stem−loop and the variable loop) were replaced by a single nucleotide, the resultant substrates had K_m values from 170 to 550 nM. Even though the D stem−loop, the anticodon stem−loop and the variable loop were not individually absolutely essential for cleavage by human RNase P, each of these domains contributed to the binding of the substrate RNA to the enzyme. However, they did not significantly affect the turnover number of the enzyme.

Table I. Kinetic parameters of cleavage of pTyr, deletion mutants of pTyr and model substrates by human RNase P

Substrate	K_m (nM)	V_{max} (nmol/min) ($\times 10^{-5}$)	V_{max}/K_m ($\times 10^{-7}$)
pTyr	10	2.9	29.00
ΔD	110	17.8	16.20
ΔAC	40	9.2	23.00
ΔAV-2	110	0.9	0.85
pATy-9j	80	14.2	17.80
pATy-1A	170	0.7	0.38
pATy-1C	420	1.6	0.38
pATy-1U	550	0.7	0.13

V_{max} is the value obtained with 0.5 μl (0.6 U) of human RNase P (see Materials and methods).

Human RNase P determines the site of cleavage of ptRNAs by measuring the length of the helix formed by the acceptor and T stems

With two exceptions, namely the precursors to $tRNA^{His}$ and $tRNA^{Secys}$ in bacteria which have 8 bp in their acceptor stems (Orellana *et al.*, 1986; Burkard and Soll, 1988), cleavage by RNase P of ptRNAs yields mature tRNAs with 7 bp in their respective acceptor stems. We do not yet understand how the exact determination of the length of the acceptor stem is made. RNase P from *Xenopus laevis* determines its cleavage site by measuring the distance along the acceptor stem of the tRNA domain in the substrate (Carrara *et al.*, 1989), but RNase P from *E.coli* does not appear to 'measure' in this manner (Svard and Kirsebom, 1992). To investigate the mechanism of selection of its cleavage site by human RNase P, a derivative in which the equivalent of the acceptor stem had 9 rather than 7 bp was made by inserting two extra base pairs into the acceptor stem of pTyr (Figure 3B). Insertion of 2 bp into the acceptor stem resulted in relocation of the cleavage site. A major cleavage occurred on the 5′ side of nucleotide +3, at a site two nucleotides away from and on the 3′ side of the original cleavage site at nucleotide +1, to yield an acceptor stem with 7 bp: a small fraction of the substrate was also cleaved at +2 to create an 8 bp stem (Figure 3D, lane 5). Similarly, when two extra base pairs were inserted into the T stem to extend the T stem to 7 bp (Figure 3C), the cleavage site was displaced one or two nucleotides downstream (Figure 3D, lane 8). These observations show that human RNase P determines its cleavage site by measuring the length of the extended RNA helix that consists of the acceptor stem and the T stem. By contrast, RNase P from *E.coli* cleaved both the substrate with an acceptor stem of 9 bp and the substrate with a T stem of 7 bp at the original site of cleavage in each case (Figure 3D, lanes 3, 6 and 9). Thus, the enzyme from *E.coli* does not strictly measure the length of the acceptor and T stems to locate its cleavage

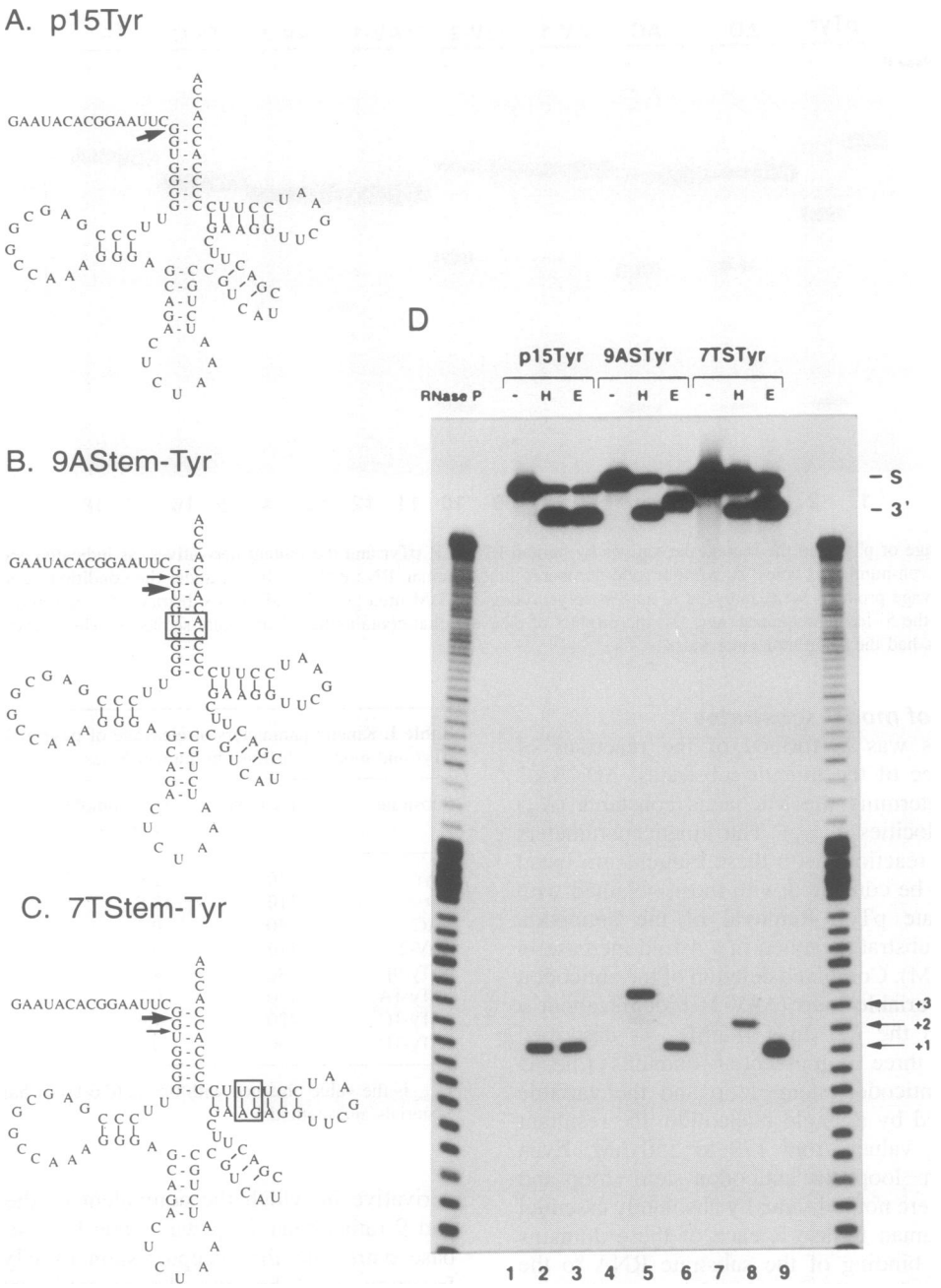

Fig. 3. Identification of sites of cleavage of pTyr and its derivatives by RNase P from HeLa cells or *E.coli*. (**A**–**C**) Sequences and secondary structures of a variant of pTyr (p15Tyr) and its derivatives in which the acceptor stem and the T stem were extended by inserting an extra 2 bp. The inserted base pairs are enclosed in boxes. Cleavage sites are indicated by arrows. (**D**) Cleavage of uniformly [α-^{32}P]GTP-labeled p15Tyr and its derivatives by RNase P from HeLa cells (H) after incubation for 60 min or by RNase P from *E.coli* (E), examined after incubation for 30 min. Cleavage sites were determined from the size of the liberated 5′ leader sequences. The bands marked +1, +2 or +3 indicate the 5′ proximal products generated by cleavage at the 5′ side of nucleotides +1, +2 or +3, respectively, of the mature tRNA sequence.

site in this substrate. These latter results agree with those of Svard and Kirsebom (1992).

A small, model substrate for human RNase P

As anticipated, a derivative of pTyr that was similar in structure to pAT-1 (McClain *et al.*, 1987), designated pATy, was not cleaved by human RNase P [Figure 4B, lanes 3 and 4; pATy is identical to the structures shown in Figure 4A (right) and Figure 5, except that it lacks the bulge at the junction of the acceptor and T stems]. To identify the minimum oligonucleotide that could retain the ability to act as a substrate, the three non-essential domains (D stem–loop, anticodon stem–loop and variable loop) were replaced with a 'bulge' of random sequence that was one to nine nucleotides in length (Figure 4A, right). This bulge was designed to allow formation of the conformational feature that was missing in pATy but was present in the parent molecule. A proposed structure of this molecule is shown in Figure 5, and this structure was confirmed for N = A (see below; Figure 9). As shown in Figure 4B, cleavage of the derivative with only one nucleotide (pATy-1N) was easily detectable (lanes 5 and 6). The derivative with nine nucleotides (pAT-9N) served as a better substrate than pATy-1N or pATy-4N (lanes 9

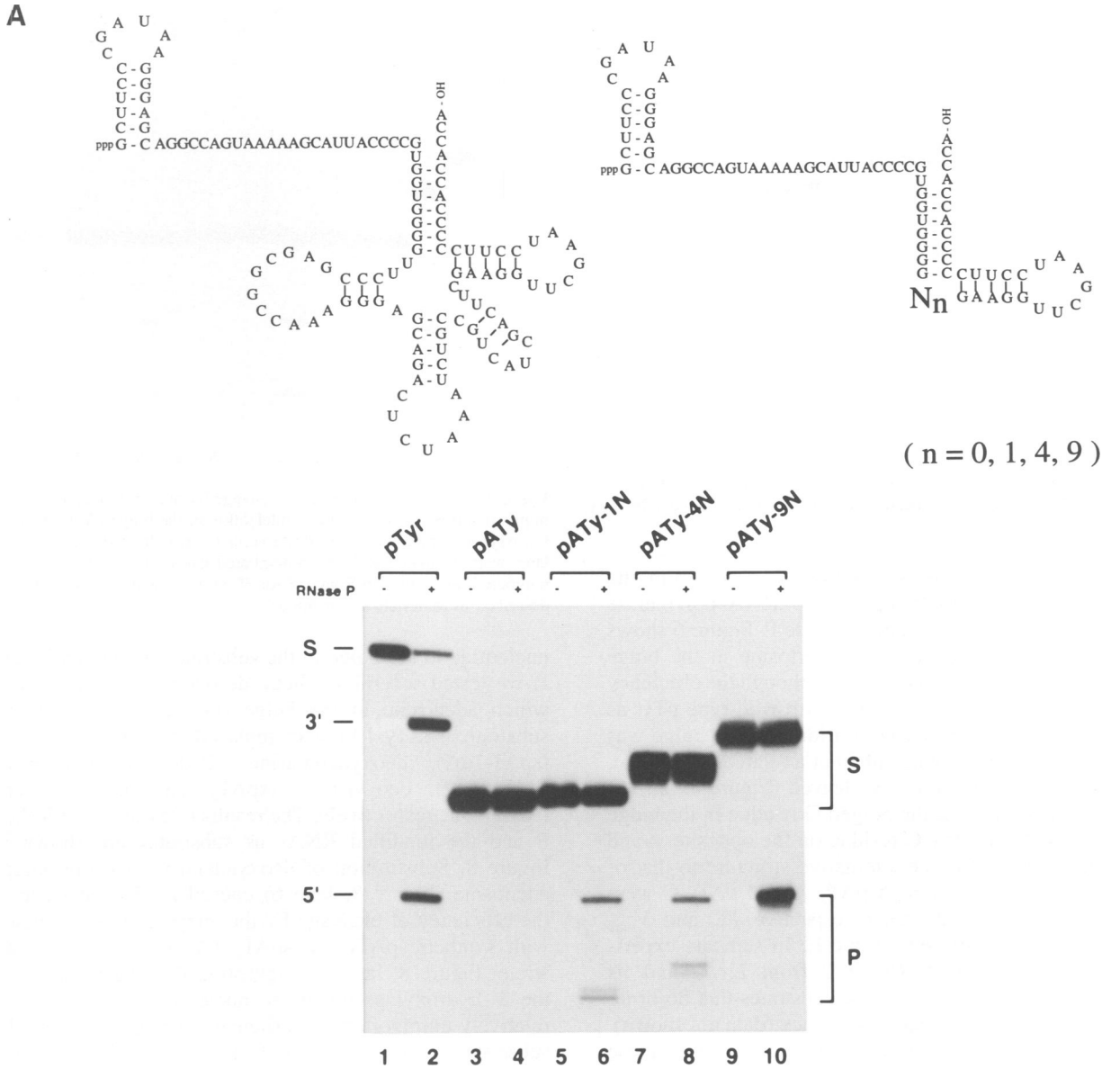

Fig. 4. Assays of cleavage of pTyr (**A**, left) and its mutant derivatives (**A**, right) in which the D stem−loop, the anticodon stem−loop and the variable loop were totally deleted (pATy) or replaced by a single nucleotide (pATy-1N), four nucleotides (pATy-4N) and nine nucleotides (pATy-9N). (**B**) pTyr and mutant variants, as indicated above each lane, were incubated with (even-numbered lanes) or without (odd-numbered lanes) human RNase P under the conditions described in Materials and methods. The cleavage products in lane 2 are indicated on the left-hand side of the panel, the others on the right-hand side of the panel. The upper bands in the region marked 'P' are the 5′ proximal cleavage products and the lower bands are the 3′ proximal cleavage products, except in lane 10 where the two products of cleavage had similar electrophoretic mobilities and appear together as an enlarged band.

and 10). The fragments of the 5′ flanking sequences released from the substrates pATy-1N, pATy-4N and pATy-9N migrated identically to that released from pTyr on the gel, indicating that human RNase P processed the novel substrates precisely at the 5′ terminus of the mature tRNA domain.

To determine if some particular sequences in the bulge region were more effective than others in providing substrate recognition features for human RNase P, we first performed simulation of evolution *in vitro* on pATy-9N in which the population of RNA molecules with a randomized bulge region was subjected to selection for ability to be cleaved by human RNase P (Yuan and Altman, 1994). Three classes of sequence were selected (Table II), each of which contained a conserved structural element, GCU, in the region corresponding to the bulge. RNA transcripts from each class of cloned sequences were tested as substrates for human RNase P. One of these, pATy-9j, served as a much better substrate than the others, being cleaved with an efficiency of ~60% of wild-type pTyr as revealed by kinetic analysis (Table I).

The DNA pool encoding pATy-1N was not subjected to any selection but was cloned into pUC19. Four individual clones, each with a different nucleotide in the bulge of the substrate (i.e. N = A, C, G or U; clones were designated pATy-1A, pATy-1C, etc.), were isolated from the pool of clones to see if a sequence preference similar to that observed with substrates that contained a bulge of nine nucleotides was apparent when the bulge contained only one nucleotide. Four RNAs, each with a different

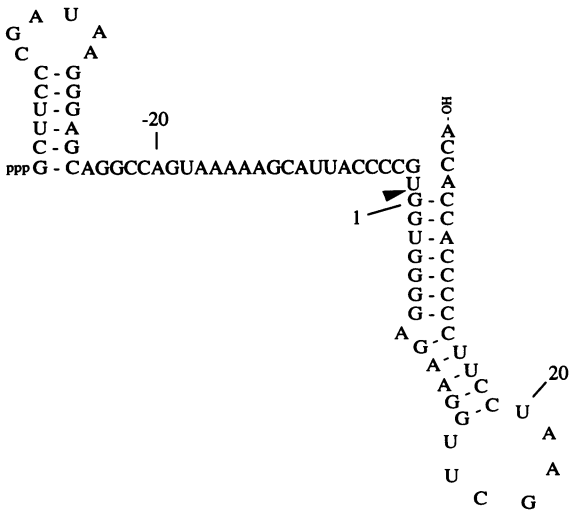

Fig. 5. Proposed secondary structure of pATy-1A, a model substrate for human RNase P. The arrowhead indicates the site of cleavage by RNase P.

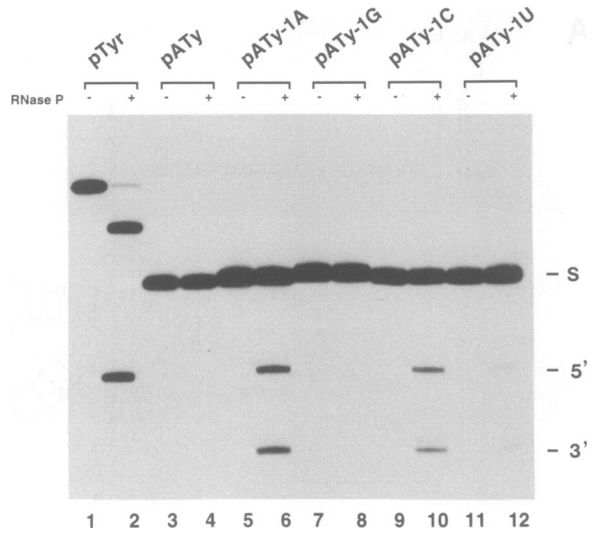

Fig. 6. Comparison of the rate of cleavage by human RNase P of minimal substrates with a single nucleotide in the bulge (A, G, C or U). pTyr and the minimal substrate variants, as indicated above each lane, were incubated with (even-numbered lanes) or without (odd-numbered lanes) human RNase P for 30 min under the conditions described in Materials and methods.

base at the bulge, were synthesized *in vitro* from the cloned plasmids by bacteriophage T7 RNA polymerase and each was exposed to human RNase P. Figure 6 shows that the RNAs with adenosine or cytosine in the bulge were substrates for human RNase P, although the efficiency of cleavage was only ~1% of that with wild-type pTyr as a substrate (Table I). RNA with uridine in the bulge was also cleaved by the enzyme, albeit at a relatively low rate. By contrast, pATy-1G was not cleaved (Figure 6, lanes 7 and 8), perhaps because the bulged G residue in the bulge could pair with a nearby C residue on the opposite strand of the helix, generating an alternative structure to that of the regular hairpin. Although pATy-A and pATy-C were equally good substrates, their respective K_m and V_{max} values were quite different (Table I). In separate experiments, we showed that RNase P from *E.coli* and its catalytic RNA subunit can cleave substrates that do or do not contain a bulge with equal efficiency (data not shown).

To confirm that the nature of the reaction with these small substrates was similar to that with the parent ptRNA, we examined the 'measuring' feature of the human enzyme. We constructed a new derivative in which the equivalent of the acceptor stem in pATy-1A was extended to 9 bp by inserting two extra base pairs in the acceptor stem. This derivative was designated 9AStem-ATy-1A (Figure 7A). As with 9AStemTyr, which was a derivative of pTyr with an extended acceptor stem of 9 bp, the cleavage site in 9AStemATy-1A was relocated to position +3, two nucleotides downstream from the normal site of cleavage, +1 (Figure 7B, lane 6). These results indicate that human RNase P determines the site of cleavage in both model and ptRNA substrates by measuring the length of the elongated RNA helix that consists of the acceptor stem and the T stem of the ptRNA.

Several features of the nucleotide in the bulge are critical for recognition by the human enzyme

Because the nucleotide in the bulge is absolutely essential for recognition of substrates by human RNase P, an important aspect of the interaction of the enzyme with its substrates must occur at the junction of the acceptor and T stems. To characterize the interaction between the nucleotide in the bulge in the substrate and human RNase P, we tested several synthetic derivatives of pATy-1A in which adenosine in the bulge (in the parent synthetic substrate, spATy-1A) was replaced by deoxyadenosine (spAT-1dA), deoxynebularine (2′-deoxyribosyl-purine: spATy-1dP), deoxyribose (spATy-1dS) or riboinosine (spATy-1I), respectively. The results of assays with RNase P and the modified RNAs as substrates are shown in Figure 8. Substitution of deoxyadenosine for the bulged adenosine (Figure 8, lane 6) caused a 15% decrease in the efficiency of cleavage by the enzyme (in comparison with synthetic pATy-1A, spATy-1A, as a standard substrate; Figure 8, lane 4), suggesting that the presence of the 2′-hydroxyl group in the nucleotide in the bulge is relatively unimportant for efficient cleavage of the model substrate by human RNase P. Removal of the 6-amino group of adenine in the bulge by replacing a deoxyadenosine with deoxynebularine (2′-deoxyribosyl-purine) brought about a further 2-fold decrease in the rate of cleavage (lane 8); replacement of A by I resulted in another 3-fold decrease in the rate of the reaction (lane 12). These results, together with the observation that pATy-1A and pATy-1C were better substrates than pATy-1U, emphasize the importance of the amino group in the nucleotide in the bulge in recognition and interaction between enzyme and substrate. When the nucleotide in the bulge was replaced by deoxyribose alone, the derivative (spAT-1dS; lane 10) could still be cleaved at a rate similar to the rate of cleavage of spATy-1dP by human RNase P (lane 8), an indication that maintenance of proper spacing along the phosphodiester chain became of paramount importance in substrate recognition in the absence of a base at the position of the nucleotide in the bulge.

To determine whether the single-nucleotide bulge renders the bases in the stem more accessible than they would be in an uninterrupted helix (Steitz, 1993), we used modification by diethylpyrocarbonate (DEPC) to examine the accessibility to reactants in solution of the region near the bulge in our model substrates. pATy and pATy-1A

Table II. Sequences of the 'tRNA' domain of substrates for human RNase P selected by simulation of evolution *in vitro*

Sequence			
Group 1	1	8	17 44
pATy-9a	GGUGGGG	**UUCGCUGUU**	GAAGGUUCGAAUCCUUCCCCCACCACCA
pATy-9b	GGUGGGG	**UUCGCUGAU**	GAAGGUUCGAAUCCUUCCCCCACCACCA
pATy-9c	GGUGGGG	**UUCGCUGUC**	G**G**AGGUUCGAAUCCUUCCCCCACCACCA
pATy-9d	GGUGGGG	**UUCGCUGAU**	GAAGGUUCGAAUCCUUCCCCCACCACCA
pATy-9e	GGUGGGG	**UUCGCUGUU**	GAAGGUUCGAAUCCUUCCCCCACCACCA
Group 2			
pATy-9f	GGUGGGG	**CCUGCUGUC**	G**G**AGGUUCGAAUCCUUCCCCCACCACCA
pATy-9g	GGUGGGG	**CCUGCUGUC**	G**G**AGGUUCGAAUCCUUCCCCCACCACCA
pATy-9h	GGUGGGG	**CCUGCUGUC**	GA**G**GGUUCGAAUCCUUCCCCCACCACCA
pATy-9i	GGUGGGG	**CCUGCUGAA**	GA**G**GGUUCGAAUCCUUCCCCCACCACCA
Group 3			
pATy-9j	GGUGGGG	**CCAGCUCCU**	GAAGGU**G**CGAAUCCUUCCCCCACCACCA
pATy-9k	GGUGGGG	**CCAGCUCCU**	G**G**AGGU**G**CGAAUCCUUCCCCCACCACCA
pATy-9l	GGUGGGG	**CCAGCUCCU**	GAAGGU**G**CGAAUCCUUCCCCCACCACCA

Numbering starts from the first nucleotide at the 3′ side of the site of cleavage by RNase P. The region of the randomized bulge of nine nucleotides is indicated in bold type. Bold underlined nucleotides are base substitutions that appeared as a consequence of the PCR amplification method.

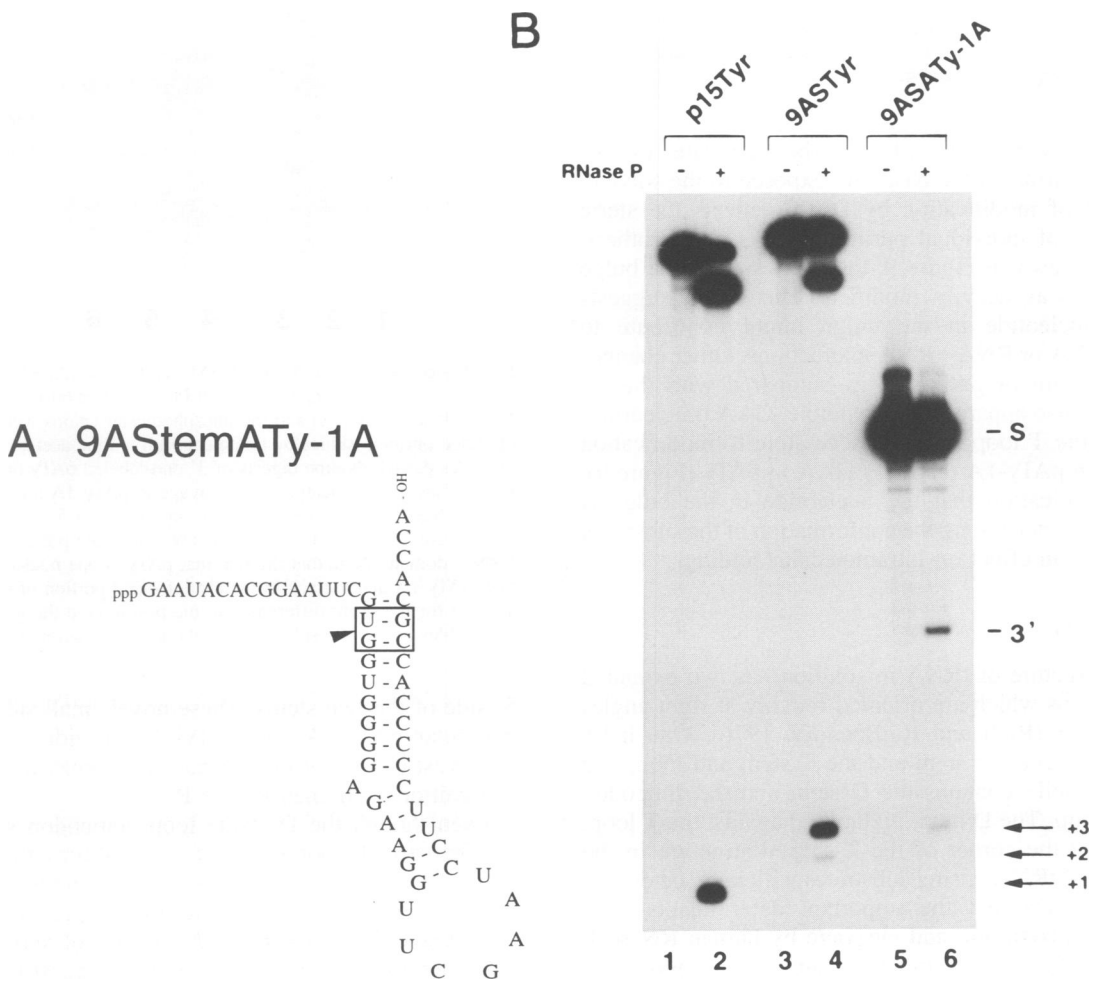

Fig. 7. Identification of sites of cleavage of a derivative of pATy-1A in which the acceptor stem of pATy-1A had been extended by inserting an extra 2 bp to yield 9AStemATy-1A, as shown in (**A**). The cleavage site is indicated by an arrow. (**B**) Uniformly [α-^{32}P]GTP-labeled p15Tyr (lane 2), 9AStemTyr (lane 4) and 9AStemATy-1A (lane 6) were subjected to digestion with RNase P from HeLa cells for 60 min as described in Materials and methods. Cleavage sites were determined from the size of the liberated 5′ leader sequence (see the legend to Figure 3D). Odd-numbered lanes show results of control experiments without RNase P.

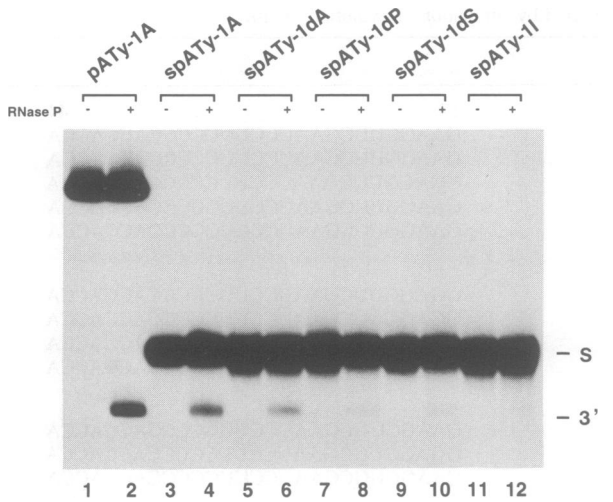

Fig. 8. Effects of variety of nucleotide analogs at the bulge of pATy-1A on cleavage by human RNase P. 3′ [^{32}P]pCp-labeled pATy-1A (lanes 1 and 2), spATy-1A (lanes 3 and 4), spATy-1dA (2′-deoxyadenosine bulge, lanes 5 and 6), spATy-1dP (2′-deoxyribosyl-purine bulge, lanes 7 and 8), spATy-1dS (2′-deoxyribose bulge, lanes 9 and 10) and spATy-1I (riboinosine bulge, lanes 11 and 12) were incubated with (even-numbered lanes) or without (odd-numbered lanes) human RNase P under the conditions described in Materials and methods. The cleavage products were analyzed in a 12% polyacrylamide−7 M urea gel. The smallest (5′ proximal) products of cleavage ran off the gel.

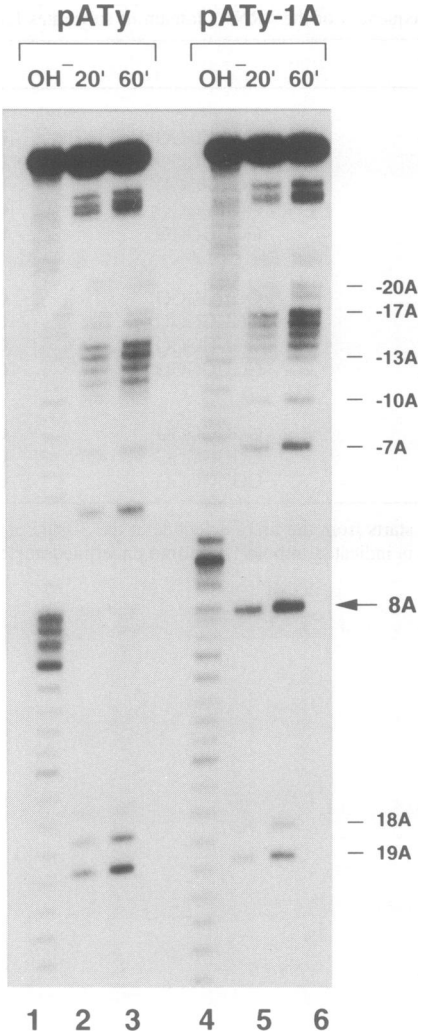

Fig. 9. Accessibility to DEPC of pATy and pATy-1A. RNAs labeled at their 3′ termini were treated with DEPC for 20 or 60 min (see Materials and methods) and the phosphodiester chains were cleaved at modified purine residues by treatment with aniline acetate. OH^- indicates partial alkaline digests of 3′ end-labeled pATy or pATy-1A RNA. Sites of modification and cleavage in pATy-1A are indicated by arrows. Negative numbers indicate positions in the 5′ leader sequence (see Figure 5) and the remaining numbers indicate positions in the 'tRNA' domain. Note that the fact that pATy is one nucleotide shorter than pATy-1A and a crack in the top left-hand portion of the gel account for the slight differences in the positions in the gel of nucleotides in the two substrates as shown in the autoradiograph.

were treated with DEPC, which carboxyethylates the N7 position of purines if the bases are exposed to the solvent. The extent of modification by DEPC reflects the steric accessibility of individual purines (Weeks and Crothers, 1993). As shown in Figure 9, the adenosine in the bulge of pATy-A was heavily modified. This result suggests that the nucleotide in the bulge might contribute to protein−RNA or RNA−RNA interactions. Other changes in the structure of pATy-1A, as compared with that of pATy, were also apparent. For example, GAA (nucleotides 17–19) in the T loop was less accessible to modification by DEPC in pATy-1A than was GAA in pATy (Figure 9), a further indication that the nucleotide in the bulge is important in determining the conformation of the substrate, possibly via an effect on intramolecular folding.

Discussion

The 3-D structure of tRNA in solution has two extended helical regions which are oriented roughly at right angles to each other (Rich and RajBhandry, 1976). One helix contains the acceptor stem and the T stem and loop, and the second helix contains the D stem and the anticodon stem and loop. The D loop, juxtaposed against the T loop, is located at the corner of the L-shaped structure in the 3-D model of tRNA. Using substrates with large deletions, we have shown that the important determinants in a ptRNA for recognition and cleavage by human RNase P are confined to the region that contains the acceptor stem and the T stem and loop plus the junction between them. Minimal substrates for human RNase P retain only the helical segment of the acceptor stem and the T stem and loop of the usual tRNA domain. In addition, they also have a bulge of a single nucleotide at the junction on the 5′ side of the two stems. These novel small substrates, in particular pATy-1A and pATy-1C, provide new models for investigations of the mechanism of enzyme−substrate recognition by human RNase P.

Even though the D stem−loop, anticodon stem−loop and the variable loop were not essential for either recognition or cleavage by human RNase P, incremental deletions of the three domains in the tRNA precursor led to progressive increases in the K_m values of substrates that contained these deletions. These data suggest that each of these domains in ptRNAs contributes in a cumulative manner, as outlined below, to binding of the substrate by human RNase P. Each domain may have a contact point(s) with human RNase P that assists in positioning the substrate RNA at the catalytic center of the enzyme.

Table III. Characteristics of plasmid constructs used for transcription *in vitro* of pTyr and its mutant derivatives

Designation	Nature of relevant insert
pTyr	gene for *E.coli* pre-tRNA^{Tyr} (pTyr) containing a 43 nucleotide 5′ leader sequence and an 85 nucleotide mature tRNA sequence, cloned in pUC-19 with upstream T7 RNA polymerase promoter (Guerrier-Takada *et al.*, 1989)
ΔD	derivative of pTyr in which the D stem−loop of 17 nucleotides (nucleotides 10–26) was deleted from pTyr
ΔAC	derivative of pTyr in which the anticodon stem−loop of 17 nucleotides (nucleotides 28–44) was deleted from pTyr
ΔV-1	derivative of pTyr in which the variable loop of 12 nucleotides (nucleotides 45–56) was deleted from pTyr
ΔV-2	derivative of pTyr in which the variable loop of 11 nucleotides (nucleotides 45–55) was deleted from pTyr
ΔAV-1	derivative of pTyr in which the anticodon stem−loop and the variable loop of 27 nucleotides (nucleotides 28–44 and 46–55) was deleted from pTyr
ΔAV-2	derivative of pTyr in which the anticodon stem−loop and the variable loop of 25 nucleotides (nucleotides 28–44 and 47–54) was deleted from pTyr
ΔTΨC	derivative of pTyr in which the TΨC stem−loop of 17 nucleotides (nucleotides 58–74) was deleted from pTyr
TL	derivative of pTyr in which a sequence of seven nucleotides (nucleotides 63–69) in the TΨC loop was replaced by GGAUCCG in pTyr
pATy	gene for a deletion mutant of pTyr, in which 50 nucleotides (nucleotides 8–57) of pTyr were deleted, cloned into pUC-19 vector at the *Eco*RI and *Sma*I sites
pATy-1A	gene for a deletion mutant in which 50 nucleotides (nucleotides 8–57) of pTyr were replaced with a single adenosine, cloned into pUC-19 vector at the *Eco*RI and *Sma*I sites
pATy-1G	50 nucleotides (nucleotides 8–57) of pTyr were replaced with a single guanosine
pATy-1C	50 nucleotides (nucleotides 8–57) of pTyr were replaced with a single cytidine
pATy-1U	50 nucleotides (nucleotides 8–57) of pTyr were replaced with a single uridine
p15Tyr	sequence corresponding to mature tRNA^{Tyr} (nucleotides 1–85) cloned into pGem-2 vector at the *Eco*RI and *Sma*I sites with upstream SP6 RNA polymerase promoter. The transcript (p15Tyr) contained a 5′ leader sequence of 15 nucleotides from the vector sequence and an 85 nucleotide mature tRNA domain
9AStemTyr	insertion of UG after nucleotide 5 and CA after nucleotide 77 in p15Tyr RNA
7TSremTyr	insertion of AG after nucleotide 59 and CU after nucleotide 72 in p15Tyr
9AStemTy-1A	a DNA fragment corresponding to 5′-GUGGUGGGGAGAAGGUUCGAAUCCUUCCCCCACCACACCA-3′, inserted into pGem-2 vector at the *Eco*RI and *Sma*I sites. The transcript, synthesized with SP6, was 55 nucleotides long

[Kahle *et al.* (1990) showed that bases in the anticodon stem of ptRNA^{Ser} and in the D stem of ptRNA^{Met} are protected from chemical modification by RNase P from yeast.] Indeed, human RNase P appears to bind to ptRNA via multiple interactions since it cleaves at a fixed distance from specific reference points in the tRNA domain of ptRNAs. The results of this study demonstrate that human RNase P determines the cleavage site in ptRNA by measuring the length of the extended RNA helix of the acceptor stem and the T stem. Cleavage of the model substrate pATy-1A also proceeded according to a 'measuring' rule. In addition, we found that a derivative of the model substrate, in which a nucleotide bulge had been relocated two nucleotides downstream from the original position, failed to serve as a substrate. Together with the observations that no cleavage occurred at position +1 and the site of the major cleavage was at position +2 when an extra 2 bp were inserted into the T stem of pTyr (7TStemTyr; Figure 3, lane 8), our data suggest that selection of the cleavage site by human RNase P involves measurements of both the length of the acceptor−T stem extended helix and the position of the bulging nucleotide within this extended helix.

The novel feature found in the model substrates for human RNase P, as compared with similar substrates for RNase P from *E.coli*, was the bulging nucleotide at the junction between the acceptor stem and the T stem. Furthermore, there was a preference for a specific nucleotide at the bulge with respect to the ability of the appropriate ptRNA derivative to serve as a substrate for human RNase P. Sequence preference of this kind was also apparent on a larger scale. If the bulge contained nine nucleotides, an element of three nucleotides was found conserved in the bulge region of all cloned sequences. Carrara *et al.* (1994) also report sequence preferences in similar model substrates for *X.laevis* RNase P.

Substrates with a bulge of adenine or cytidine (pATy-1A and pATy-1C) were 3-fold more susceptible to cleavage than a substrate with a uridine at the bulge (pATy-1U), while a substrate with a bulging G was not cleaved at all (see above). These results suggest that the 6-amino group of adenosine or the 4-amino group of cytosine in the bulge might very likely make a crucial contact with other components of the enzyme−substrate complex or might be critical for maintaining an appropriate configuration of the substrate. However, since a substrate with no base but only a deoxyribose moiety could still be cleaved, the bulge region must be able to assume a variety of conformations that can still be recognized by the enzyme even though the atomic interactions that govern this recognition may change depending on the chemical nature of the bulge.

The bulge in the small model substrate interrupts one strand of an otherwise continuous RNA helix. As a consequence, the bulge itself or other nucleotides in the helix become accessible for interactions with the enzyme. The accessibility of the bulging nucleotide and its importance in the cleavage reaction are supported by two lines of evidence: (i) the ability of a substrate that contains a deoxyribonucleotide to be cleaved by human RNase P and (ii) the results of modification experiments with DEPC.

A bulge of a single nucleotide in an RNA helix has been shown to serve as a protein binding site in several instances (for a review see Wyatt and Tinoco, 1993). A bulging adenosine residue is essential for binding of the R17 coat protein to the translational operator region in the RNA (Wu and Uhlenbeck, 1987). The binding site of the ribosomal protein L18 is a helical region interrupted by a bulging adenosine (Peattie *et al.*, 1981). The three, individually non-essential domains in ptRNAs substrates

for human RNase P can be thought of as merely part of a large bulge. The single-nucleotide bulge may provide a recognition feature akin to some aspect of tertiary folding in a wild-type ptRNA and it reveals an important difference between the enzymes from human and *E.coli* in terms of substrate recognition. A key difference in the nature of substrate recognition between the enzymes from human and *E.coli* might be the direct involvement of a protein component in substrate recognition by the human enzyme via interactions with the bulging nucleotide. If valid, our hypothesis could explain why human RNase P can function only as a ribonucleoprotein complex while the RNA component of RNase P from *E.coli* can catalyse cleavage by itself in solution.

Materials and methods

Construction of plasmids

Clones encoding deletion or substitution mutants of the precursor to $tRNA^{Tyr}$ su3 (pTyr) from *E.coli* were constructed by inserting appropriate DNA restriction fragments, synthetic double-stranded oligonucleotides, fragments of DNA obtained by the PCR or combinations thereof into pUC19 that contained a T7 promoter sequence upstream from the potential insertion site. Specifically, DNA coding for pATy-1N (N refers to a mixture of the four nucleotides, i.e. A, T, G and C) was synthesized by PCR, using pTyr DNA as template (Guerrier-Takada *et al.*, 1989), with the two primer oligonucleotides T7 (5′-GGAATTCGAAATTAATACGACTCACTATAG-3′) and AT-1N (5′-TGGTGGTGGGGGAAGGATTCGAACCTTCNCCCCACCACGGGGTAATGC-3′). pTyr DNA contains the sequence of the gene for the naturally occurring precursor to $tRNA^{Tyr}$ from *E.coli* adjacent to an upstream bacteriophage T7 promoter sequence.

After the DNA had been inserted into pUC19 at the *Eco*RI and *Sma*I sites, four individual clones were analyzed by sequencing. DNA coding for substrate pATy was synthesized and cloned in a similar manner using oligonucleotide AT-0 (5′-TGGTGGTGGGGGAAGGATTCGAACCTTCCCCCACCACGGGGTAATGC-3′) instead of oligonucleotide AT-1N in the PCR. Plasmids constructed and used in this study are shown in Table III.

Evolution in vitro

DNA coding for pATy-9N was synthesized by PCR using pTyr DNA (Guerrier-Takada *et al.*, 1989) as template with the two primer oligonucleotides T7 (see above) and AT-9N (5′-TGGTGGTGGGGGAAGGATTCGAACCTTCNNNNNNNNNCCCCACCACGGGGTAATGC- 3′). An RNA pool of transcripts of pATy-9N was prepared by transcription of the amplified DNA template with T7 RNA polymerase. Selection of substrates for human RNase P was carried out as described previously (Yuan and Altman, 1994). The oligonucleotides used for coupled reverse transcription PCR in the selection procedure were AT-3 (5′-GTAATACGACTCACTATAGAATACACGGAATTGGTGGGG-3′) and pTyr-3 (5′-GCCAAGCTTCCTGGTGGAGGGGGAAGGATTCG-3′). After six cycles of selection, the resulting double-stranded DNAs were cloned into pUC19 at the *Sma*I−*Hin*dIII site. Individual clones were selected and 12 plasmid DNAs were sequenced using Sequenase 2.0 (US Biochemicals).

Assay for RNase P activity

Human RNase P was purified from HeLa cell nuclei through DEAE−Sepharose chromatography and a glycerol gradient step, as described by Bartkiewicz *et al.* (1989). RNA substrates were transcribed *in vitro* in the presence of [α-^{32}P]GTP with T7 RNA polymerase from plasmids that had been linearized by treatment with appropriate restriction endonucleases. Reactions catalyzed by RNase P were carried out in 10 μl of 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NH_4Cl and 0.2 pmol (1000 c.p.m.) substrate RNA. Reaction mixtures were incubated at 37°C with 1 μl (1.2 U) RNase P from HeLa cells, with subsequent electrophoresis in 8% polyacrylamide−7 M urea gels.

Kinetic analysis

Cleavage of pTyr and a variety of derivatives of pTyr was examined at various concentrations of substrate both above and below the K_m for each individual substrate. Aliquots were withdrawn from reaction mixtures at regular intervals and analyzed on polyacrylamide−urea gels. Values of K_m and V_{max} were obtained from Lineweaver−Burk double-reciprocal plots.

Accessibility to DEPC

Modification of RNA by DEPC was performed as described by Weeks and Crothers (1993). RNA labeled at its 3′ terminus with [^{32}P]pCp was treated with 10% DEPC in 25 mM HEPES (pH 7.5), 70 mM NaCl, 1 mM EDTA and 10 μg/ml tRNA from *E.coli*. The reaction was incubated for either 20 or 60 min at room temperature. Cleavage of the phosphodiester chain at modified purines was induced by treatment with 1 M aniline (pH 4.5). Samples were then suspended in 1× RNA dye solution (4.5 M urea, 10 mM EDTA, 0.125 mg/ml bromophenol blue, 0.125 mg/ml xylene cyanol) and loaded onto a 15% sequencing gel.

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References

Altman,S., Gold,H.A. and Bartkiewicz,M. (1988) In Birnstiel,M. (ed.), *Structure and Function of Small Ribonucleoproteins.* Springer Verlag, Berlin, Germany, pp. 183–195.
Bartkiewicz,M., Gold,H. and Altman,S. (1989) *Genes Dev.*, **3**, 488–499.
Burkard,U. and Soll,D. (1988) *Nucleic Acids Res.*, **16**, 11617–11624.
Carrara,G., Calandra,P., Fruscoloni,P., Doria,M. and Tocchini-Valentini,G.P. (1989) *Cell*, **58**, 37–45.
Carrara,G., Calandra,P., Fruscoloni,P. and Tocchini-Valentini,G. (1994) *Proc. Natl Acad. Sci. USA*, in press.
Forster,A.C. and Altman,S. (1990) *Cell*, **62**, 407–409.
Guerrier-Takada,C. and Altman,S. (1984) *Science*, **233**, 285–286.
Guerrier-Takada,C., Gardiner,K., Marsh,T., Pace,N. and Altman,S. (1983) *Cell*, **35**, 849–857.
Guerrier-Takada,C., Lumelsky,N. and Altman,S. (1989) *Science*, **246**, 1578–1584.
Kahle,D., Wehmeyer,U. and Krupp,G. (1990) *EMBO J.*, **9**, 1929–1937.
McClain,W.H., Guerrier-Takada,C. and Altman,S. (1987) *Science*, **238**, 527–530.
Orellana,O., Cooley,L. and Soll,D. (1986) *Mol. Cell. Biol.*, **6**, 525–529.
Peattie,D.A., Douthwaite,S., Garrett,R.A. and Noller,H.F. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 7331–7335.
Rich,A. and RajBhandary,U.L. (1976) *Annu. Rev. Biochem.*, **45**, 805–860.
Steitz,T.A. (1993) In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 219–238.
Svard,S.G. and Kirsebom,L.A. (1992) *J. Mol. Biol.*, **227**, 1019–1031.
Weeks,K. and Crothers,D. (1993) *Science*, **261**, 1574–1577.
Wu,H.-N. and Uhlenbeck,O.C. (1987) *Biochemistry*, **26**, 8221–8227.
Wyatt,J.R. and Tinoco,I. (1993) In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 465–496.
Yuan,Y. and Altman,S. (1994) *Science*, **263**, 1269–1273.
Yuan,Y., Hwang,E.-S. and Altman,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8006–8010.

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