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Multiple tRNA attachment sites in prothymosin α

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Abstract A covalent complex formed by bacterial tRNAs and prothymosin α, an abundant acidic nuclear protein involved in proliferation of mammalian cells, upon production of the recombinant rat protein in *Escherichia coli* cells was studied. Several tRNA attachment sites were identified in the prothymosin α molecule using a combination of deletion analysis of prothymosin α and site-specific fragmentation of the protein moiety of the prothymosin α-tRNA complex. The electrophoretic mobilities of the tRNA-linked prothymosin α and its derivatives are consistent with one tRNA molecule attached to one prothymosin α molecule, thus suggesting that alternative tRNA linking to one of several available attachment sites occurs. The possible effect of tRNA attachment on the nuclear uptake of prothymosin α is discussed.

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Key words: Prothymosin α; Transfer RNA; Nuclear localization signal; *Escherichia coli*

1. Introduction

Ribonucleoproteins with covalently linked RNA and protein moieties are widespread in the viral world. About 25% of eukaryotic RNA viruses contain a protein called VPg (for viral protein genome-linked) attached to the 5′ terminus of their genomic RNA through a phosphodiester linkage (for reviews see [1,2]). VPg was proved to play a crucial role in the replication cycle of these viruses [3]. However, only a few cellular covalent RNA-protein complexes are known. The best characterized example is that formed by the tumor suppressor protein p53 and 5.8S rRNA [4]. Another nuclear protein involved in proliferation of the mammalian cells, prothymosin α (ProTα), was reported to link a short RNA fragment of an unknown origin in mouse Krebs 2 cells [5,6]. Recently, the ability of ProTα to link an RNA was mimicked in *Escherichia coli* cells producing recombinant mammalian ProTα [7]. In this case, the RNA moiety of the complex was characterized and proved to contain a set of tRNAs, including $tRNA^{Lys}$, $tRNA_3^{Ser}$, $tRNA_2^{Ile}$ and $tRNA_m^{Met}$ [7]. As in mouse cells, the ProTα-linked bacterial tRNAs possess free 3′ termini and 5′ termini that are blocked, evidently by ProTα. A tRNA attachment site was mapped in the carboxy-terminal half of ProTα [7] where its nuclear localization signal resides [8], raising the possibility that tRNA linking could influence the subcellular localization of the protein. Here, by using a deletion mutagenesis analysis of ProTα, we present evidence that there are several tRNA linking sites within the ProTα molecule. Moreover, a number of ProTα fragments appear to be competent in tRNA attachment.

2. Materials and methods

2.1. Plasmid constructions

Production of carboxy-terminal $(His)_6$-tagged rat ProTα and its derivatives in *E. coli* cells was driven by pQE60 and pQE70 vectors (QIAGEN) carrying corresponding inserts of the protein encoding regions of rat ProTα cDNA. Construction of the parental pQN3 plasmid encoding the full-length rat ProTα and the plasmid encoding ProTα mutant I was described previously [7]. For constructing a plasmid encoding ProTα mutant III, pQN3 was digested with *Dde*I, blunt-ended with the Klenow fragment in the presence of dNTP and subsequently cleaved with *Eco*RI. A 275 bp long DNA fragment was inserted in the pQE70 vector between the S1 nuclease blunt-ended *Bgl*II site and *Eco*RI site, producing the pQWC plasmid.

A plasmid encoding ProTα mutant VIII was obtained by ligating a 310 bp long *Sty*I-*Hin*dIII fragment of pQN3 into pQE60 digested with *Nco*I and *Hin*dIII. Transformants with the restored initiation ATG codon were selected by screening for production of mutated ProTα.

For constructing the ProTα mutant II, PCR was performed on pQN3 with the ProTα-specific primer H (5′-TGGTATCGACATCGTCATCCTC-3′) and pQE promotor primer (QIAGEN). The resultant PCR product was digested with *Taq*I, blunt-ended with S1 nuclease, cleaved with *Eco*RI and inserted into the pQE70 vector between the S1 nuclease blunt-ended *Bgl*II site and *Eco*RI site. Sequencing of the vector-insert boundary revealed that S1 nuclease has removed three extra nucleotides from the ProTα cDNA resulting in the ProTα Asp-98 codon immediately preceding the $(His)_6$ encoding sequence.

A cDNA fragment encoding ProTα mutants V and VII was obtained by PCR on pQWC with the ProTα-specific primer ΔT (5′-GACCATGGGAAGAGACGCCCCT-3′) and pQE reverse sequencing primer (QIAGEN). To construct ProTα mutant VII, the PCR product was hydrolyzed with *Nco*I and *Hin*dIII and inserted into a similarly digested pQE60 vector. To construct ProTα mutant V, the same PCR product was digested with *Acy*I, partially filled-in with the Klenow fragment in the presence of dCTP, blunt-ended with S1 nuclease and cleaved with *Hin*dIII. The resultant DNA fragment was inserted in the pQE60 vector between the filled-in *Nco*I site and *Hin*dIII site, producing the pQAc plasmid.

For constructing ProTα mutant VI, a megaprimer was obtained by PCR on pQAc with the ProTα-specific primer M (5′-TCCTGCTCCCCATTTTCCTCATT-3′) and pQE promotor primer. Then, PCR on pQN3 with the megaprimer and primer H was performed, the resulting product was cleaved with *Eco*RI and inserted in the pQE70 vector between the *Eco*RI site and filled-in *Bgl*II site.

A cDNA fragment encoding ProTα mutant IX was obtained by a PCR on pQN3 with the ProTα-specific primer α1 (5′-TCCTCTGCCTCCTCCACAAC-3′) and pQE promotor primer. The resulting PCR product was hydrolyzed with *Eco*RI and inserted in the pQE70 vector between the *Eco*RI site and S1 nuclease blunt-ended *Bgl*II site.

To obtain ProTα mutant IV containing the enterokinase cleavage site, a megaprimer was synthesized by PCR on pQN3 with the mutagenic primer D_4K (5′-GGAGATGAtGATGAtaAAGCT-3′) (mutations are indicated by lowercase letters) and pQE reverse sequencing primer. This megaprimer was used, in combination with a pQE promotor primer, to amplify a DNA fragment encoding the full-length mutated ProTα. The PCR product was cleaved with *Eco*RI and *Hin*dIII and inserted into similarly digested pQE70 vector.

The structure of all constructs was confirmed by sequencing.

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Abbreviations: ProTα, prothymosin α; bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis

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2.2. Isolation of prothymosin α-tRNA complexes

ProTα mutants and their complexes with tRNA were isolated from *E. coli* JM109 cells carrying appropriate plasmids as described for full-length ProTα [7]. Procedures for radiolabelling of the ProTα-linked tRNA and fractionation of the ProTα-tRNA complexes and tRNA-peptides by Ni-NTA affinity chromatography and polyacrylamide gel electrophoresis (PAGE) were reported previously [7].

2.3. Proteolytic hydrolysis of prothymosin α-tRNA complexes

[^{32}P]-labelled ProTα-tRNA complexes were treated with 1 μg of trypsin in 20 μl of a buffer containing 50 mM Tris-HCl, pH 8.0, 1 M guanidine-HCl and 5 μg free ProTα used as a carrier. After incubation at 37°C for 1 h, the sample was diluted with 200 μl buffer B (6 M urea, 50 mM NaCl, 10 mM Tris-HCl, pH 8.0) and tRNA-linked tryptic peptides were fractionated by Ni-NTA-agarose chromatography and 8% PAGE.

Hydrolysis of the [^{32}P]-labelled ProTα mutant IV-tRNA complex with 0.15 U bovine enterokinase (kindly provided by A. Mikhailova) was in 15 μl of a buffer containing 10 mM Tris-HCl, pH 8.0, 10 μg tRNA and 15 μg ProTα used as carriers. The sample was incubated at 37°C overnight, diluted with 200 μl buffer B and applied on the Ni-NTA resin (QIAGEN) as described [7].

3. Results

3.1. Evidence for the tRNA attachment site within the amino-terminal region of ProTα

Production of mammalian ProTα in a heterologous *E. coli* environment was previously shown to result in tRNA attachment to this small (111 amino acid residues long) highly acidic protein (Fig. 1A) mimicking the formation of a ProTα-RNA complex observed in mouse cells [7]. A tRNA attachment site located close to the carboxy-terminus of ProTα was mapped. Moreover, the carboxy-terminal ProTα fragment (residues 86–111, mutant I, Fig. 1B) itself, when synthesized in *E. coli* cells, retained the ability of the full-length protein to link to tRNA [7]. To further delineate the tRNA linking region in ProTα, we constructed a set of rat ProTα deletion mutants listed in Fig. 1B, overproduced these proteins in *E. coli* cells and assayed them for the ability to link tRNA. For the purpose of purification and analysis of the ProTα-tRNA complex, we fused rat ProTα and its derivatives with a $(His)_6$ tag at their carboxy-termini, a modification shown previously not to influence tRNA linking by ProTα [7].

First, we assayed two ProTα mutants with progressively greater deletions of the carboxy-terminal region of the protein: mutant II (1–98) and mutant III (1–83) (Fig. 1B). These proteins and their putative complexes with tRNA were isolated from bacterial cells according to the previously established protocol [7] including cell boiling in 10% SDS, hot phenol deproteinization exploiting the property of the ProTα-tRNA complex and free ProTα to retain in the aqueous phase [5,10,11] and denaturing Ni-NTA affinity chromatography in the presence of 6 M urea. This resulted in a preparation containing ProTα and ProTα-tRNA covalent complex free from contaminating cellular proteins and RNAs. Because the amount of the complex formed is usually small relatively to the total amount of ProTα produced in bacterial cells [7], 3′ end labelling of the ProTα-linked tRNA with [^{32}P]pCp and T4 RNA ligase was performed. Fractionation of the labelled compounds by urea-PAGE revealed that both ProTα mutants II and III retained the ability to attach tRNA (Fig. 2A). As with the full-length ProTα, proteinase K treatment of the complexes formed by the II and III mutants resulted in the formation of the tRNA length

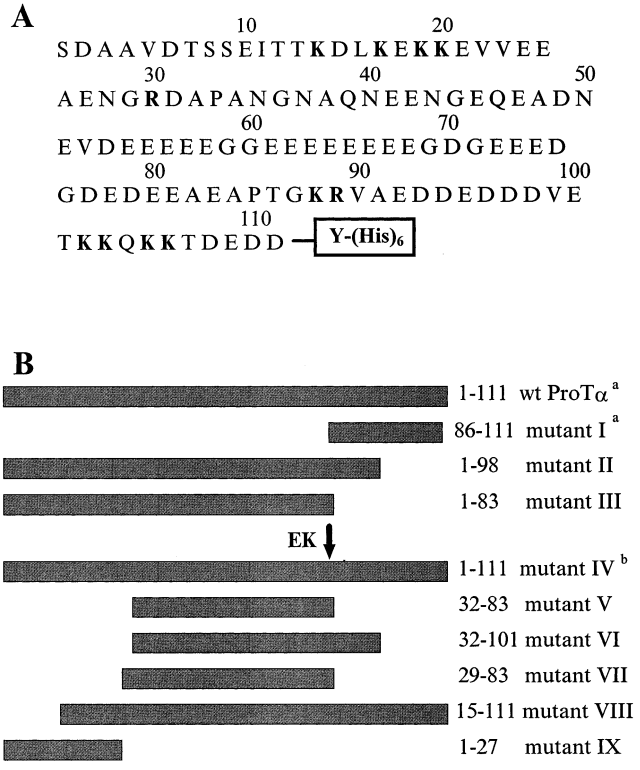


Fig. 1. (A) Primary structure of rat ProTα [9] with the carboxy-terminal hexahistidine tag. Basic amino acid residues employed for protein fragmentation are in bold. (B) ProTα derivatives assayed for tRNA linking and localization of tRNA attachment sites. Construction of these proteins was described previously [7]. The enterokinase (EK) cleavage site introduced in the ProTα molecule at position 81 is indicated by an arrow.

radiolabelled RNA moiety, while digestion with RNase A eliminated the label, as expected (Fig. 2A).

The ability of the deletion mutant III to link tRNA was particularly surprising because this truncated protein completely lacked the carboxy-terminal region of ProTα capable of autonomous tRNA linking, as demonstrated earlier (mutant I, residues 86–111, Fig. 1B) [7]. We conclude therefore that, apart from the carboxy-terminal tRNA linking site, additional site(s) of tRNA attachment do occur in the ProTα molecule.

To verify that both this additional and the carboxy-terminal tRNA attachment sites are indeed present in the full-length ProTα and did not emerge as a consequence of deletion mutagenesis, a recognition sequence (DDDDK) for a site-specific endoproteinase (enterokinase) was introduced into the full-length ProTα molecule at position 81 (mutant IV, Fig. 1B), so that the products of the enterokinase cleavage should correspond to the sum of deletion mutants I and III. The ProTα mutant IV-tRNA complex containing end-labelled RNA was digested with enterokinase and analyzed by fractionation on a Ni-NTA resin with subsequent gel electrophoresis (Fig. 2B). Endoproteinase treatment resulted in the formation of the two RNA-peptides designated as Nt and Ct in Fig. 2B. Enterokinase cleavage of the ProTα moiety of the complex was only partial which accounts for the significant amount of the uncleaved complex (lane 3). Both Nt and Ct liberated tRNA upon treatment with proteinase K (not shown). The larger

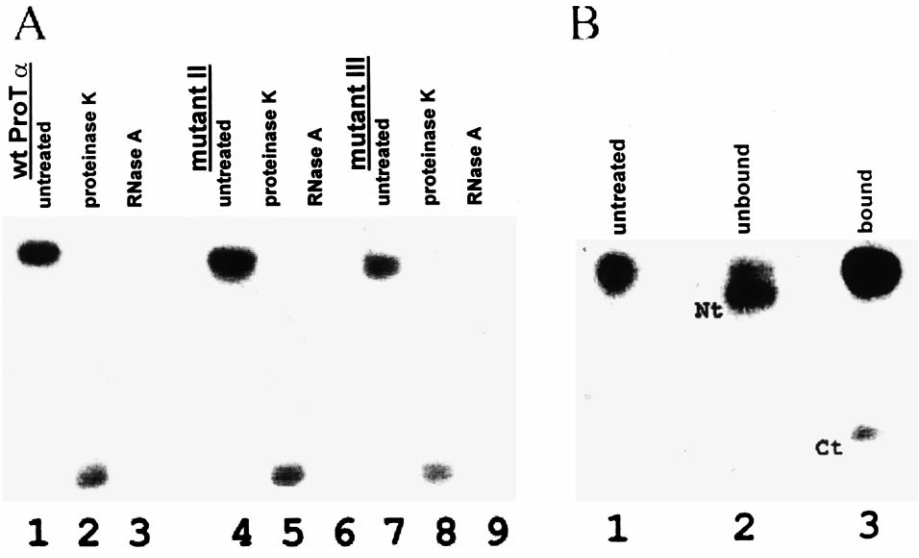


Fig. 2. tRNA attachment is not limited to the carboxy-terminal portion of ProTα. (A) ProTα mutants II and III with progressively greater deletions of the carboxy-terminal region of the protein are still capable of tRNA attachment. [^{32}P]-labelled ProTα-tRNA complexes were resolved by electrophoresis in a 8% polyacrylamide/7 M urea gel before (lanes 1, 4, 7) or after the treatment with proteinase K (lanes 2, 5, 8) or RNase A (lanes 3, 6, 9). (B) [^{32}P]-labelled ProTα mutant IV-tRNA complex (lane 1) was hydrolyzed with enterokinase, fractionated by Ni-NTA affinity chromatography and analyzed in an 8% denaturing polyacrylamide gel. Lane 2, unbound fraction; lane 3, Ni-NTA bound fraction. Nt and Ct denote the tRNA-linked amino- and carboxy-terminal ProTα fragments, respectively.

RNA-peptide Nt lacked the $(His)_6$ tag, as it was unable to bind to the Ni-NTA resin (Fig. 2B) and thus, it corresponds to the amino-terminal portion of ProTα (residues 1–81). The shorter RNA-peptide Ct was bound by this resin and could be eluted with imidazole indicating the presence of the $(His)_6$ tag. Ct thus corresponds to the carboxy-terminal ProTα fragment (residues 82–111). We conclude that both the amino- and carboxy-terminal tRNA attachment sites are functional in the context of full-length ProTα. Judging by the relative intensities of the Nt and Ct bands, the amino-terminal site may be utilized more frequently for the tRNA linking than the carboxy-terminal site.

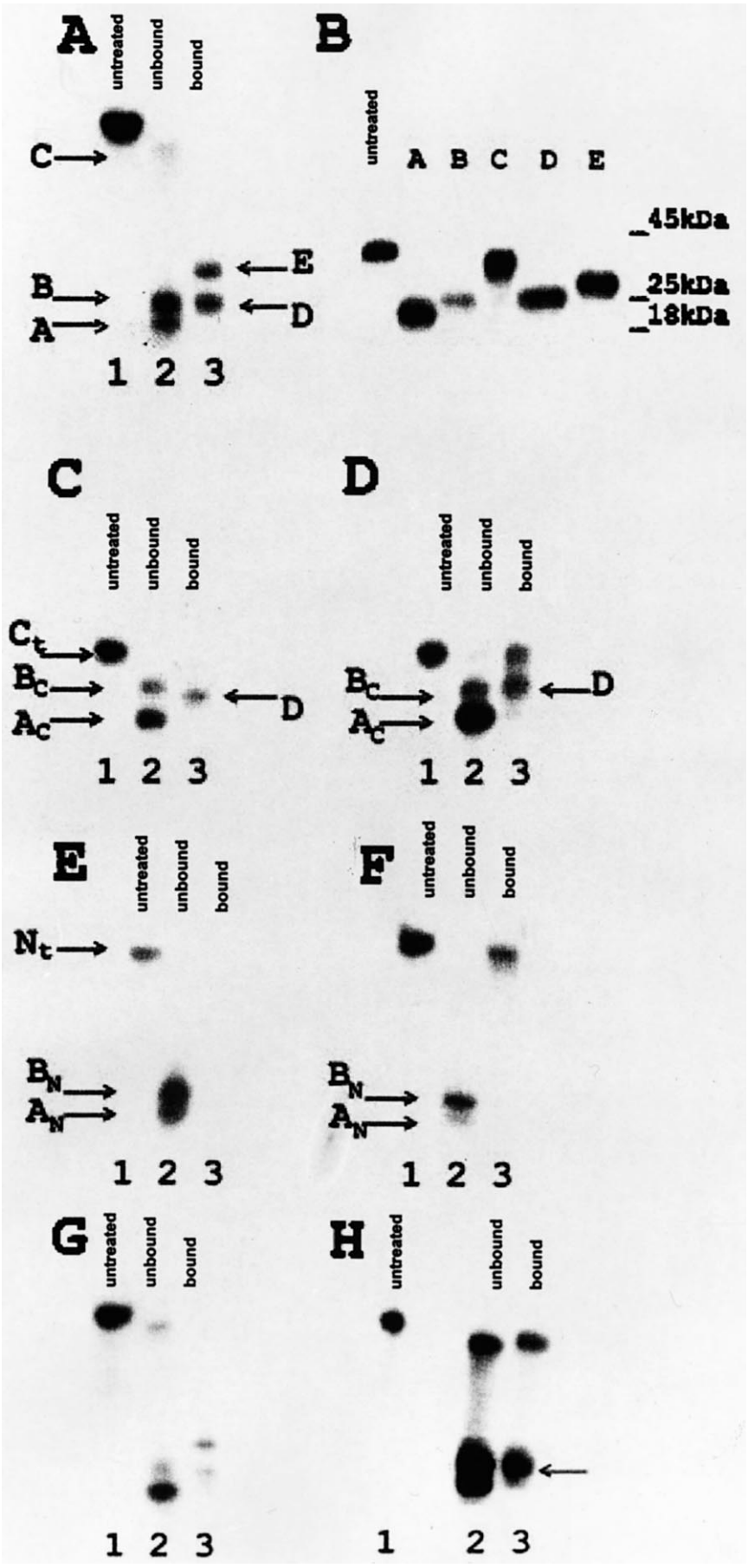
To further confirm our identification of Nt and Ct as the RNA-linked amino- and carboxy-terminal portions of ProTα, respectively, and to narrow down the regions of ProTα responsible for tRNA linking, the gel-purified ^{32}P-labelled Nt and Ct RNA-peptides were subjected to hydrolysis with trypsin. The resultant labelled tRNA-linked tryptic peptides were analyzed for the presence of the carboxy-terminal (His_6) tag by Ni-NTA chromatography and subsequently fractionated by PAGE for the estimation of the size of the attached peptide(s). For comparison, the same type of analysis was performed on the tRNA-linked wild-type ProTα and on corresponding complexes formed with ProTα mutants I and III.

In the wild-type ProTα-tRNA complex, three untagged tRNA-peptide species (bands A, B and C) and two $(His)_6$-tagged species (bands D and E) were observed (Fig. 3A). However, repeated treatment with trypsin converted tRNA-peptide C into A and B and tRNA-peptide E into D, A and B (not shown), indicating that tRNA partially protected the protein moiety of the complex from proteolytic digestion. The electrophoretic mobilities of the RNA-peptides correlated with the length of the peptide moieties in this electrophoretic system. This result was verified by re-electrophoresis of the individual RNA-peptides A–E in a SDS-containing polyacrylamide gel (Fig. 3B). Besides, proteinase K digestion of these individual RNA-peptides led to the formation of the RNA comigrating with authentic tRNA (not shown), demonstrating the uniform length of the RNA moieties of the RNA-peptides under study. Importantly, from the electrophoretic mobilities of the intact complex and its RNA moiety in the SDS-containing gel, which corresponded to those of 40 kDa and 20–25 kDa proteins, respectively, it appears likely that only one tRNA is linked to one ProTα molecule. In case of occurrence of multiple tRNA attachment sites in ProTα, this result implies that the population of ProTα-tRNA complex includes two types of complexes, those with a tRNA molecule linked either to the amino- or to the carboxy-terminus of ProTα.

The patterns of tryptic tRNA-peptides generated from the complexes formed by Ct enterokinase cleavage product (Fig. 3C), by ProTα mutant I (Fig. 3D) and by wild-type ProTα (Fig. 3A) were very similar to each other, giving an illusion that tRNA attachment is limited to the carboxy-terminal region of ProTα. However, the tRNA-peptide patterns of the Nt and mutant III complexes (Fig. 3E and F), which were also similar to one another, revealed the presence of two short untagged RNA-peptides with mobilities similar to those derived from the carboxy-terminal region of ProTα. Thus, comigration of the untagged RNA-peptides that originated from the amino- (A_N and B_N) and carboxy- (A_C and B_C)terminal halves of ProTα masked the amino-terminal tRNA attachment site, but could be disclosed with the help of ProTα

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Fig. 3. Analysis of the tRNA-linked tryptic peptides derived from the wild-type ProTα (A and B), Ct (C), Nt (E), ProTα mutants I (D), III (F), VIII (G) and II (H). ^{32}P]-labelled ProTα-tRNA complexes (lanes 1) were digested with trypsin, fractionated by Ni-NTA chromatography and analyzed in 8% polyacrylamide/7 M urea gels (A, C–H) or in a 12% polyacrylamide/SDS gel (B). Lanes 2, unbound fractions; lanes 3, Ni-NTA bound fractions. Positions of protein molecular weight markers are indicated by the arrows in B. In H, an arrow indicates the short characteristic tRNA-linked peptide with $(His)_6$ tag.


A
untreated
unbound
bound
C
B
A
E
D
1 2 3
B
untreated
A B C D E
45kDa
25kDa
18kDa
C
untreated
unbound
bound
Ct
BC
AC
D
1 2 3
D
untreated
unbound
bound
BC
AC
D
1 2 3
E
untreated
unbound
bound
Nt
BN
AN
1 2 3
F
untreated
unbound
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BN
AN
1 2 3
G
untreated
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1 2 3
H
untreated
unbound
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1 2 3

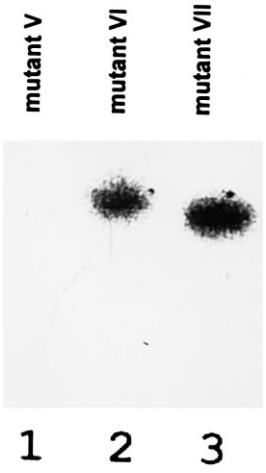


Fig. 4. The tRNA linking capacity of the central acidic portion of ProTα (mutant V, lane 1) can be restored by both the carboxy- and amino-terminal extension of this protein. Lane 2, ProTα mutant VI; lane 3, ProTα mutant VII. An autoradiogram of an 8% polyacrylamide/7 M urea gel is shown.

deletion mutants and the products of enterokinase fragmentation of ProTα.

Generation of short tRNA-linked peptides derived from the amino-terminal part of ProTα implies that tRNA attachment occurs within the first 30 amino acid residues of ProTα (see Fig. 1A for location of basic amino acids in the ProTα molecule). Moreover, the presence of tRNA-peptide A_N with an electrophoretic mobility similar to that of free tRNA is indicative for a tRNA attachment site closely flanked by the basic amino acids. The ProTα region 14–20 is thus an obvious candidate for this role. However, the fact that two (A_N and B_N) rather than one tRNA-peptide were observed might suggest either the existence of several tRNA attachment sites or, in the case of B_N, the incomplete trypsin digestion of the protein due to shielding of the susceptible bonds in ProTα by the covalently linked tRNA. In this last scenario, the tRNA-linked peptide B_N could correspond to residues 1–20 or 15–30 of ProTα (see also Fig. 5 for schematic representation of the data).

Further analysis of the amino-terminal tRNA linking was hampered by our inability to observe tRNA attachment to a small amino-terminal ProTα derivative (mutant IX, residues 1–27, Fig. 1B) (not shown). Both instability of this peptide within the cell and its inability to link tRNA could account for this result. Besides, introduction of the enterokinase cleavage site within this region (residues 24–28) led to the formation of a protein that could not be cleaved by this enzyme for an unknown reason. As an alternative approach, we constructed an amino-terminally truncated ProTα mutant (mutant VIII, Fig. 1B). This mutant was able to link tRNA, as expected (Fig. 3G). Analysis of the tRNA-peptides derived from this complex revealed that amino-terminal truncation did not result in elimination of bands A and B (Fig. 3G, lane 2), due to the above mentioned masking of these tRNA-peptides by the co-migrating tRNA-peptides A_C and B_C derived from the carboxy-terminal portion of the ProTα molecule. It did result, however, in the increased intensity of band A (compare with Fig. 3A), which may reflect conversion of the B_N tRNA-peptide into a shorter form co-migrating with A.

3.2. Evidence for two tRNA attachment sites within the carboxy-terminal region of ProTα

Realization of the fact that multiple tRNA attachment sites exist in ProTα prompted us to investigate the carboxy-terminal tRNA linking in more detail. Analysis of the tryptic tRNA-peptides derived from the wild-type ProTα-tRNA, ProTα mutant I-tRNA and Ct complexes revealed a number of bands (A_C, B_C, and D) (Fig. 3A, C, D). Again, the presence of a very short tRNA-peptide A_C with an electrophoretic mobility similar to that of free tRNA is indicative of a tRNA attachment site closely surrounded by lysine residues. The larger tRNA-linked peptides B_C and D might then be the products of incomplete hydrolysis of the protein moiety with trypsin due to shielding of the scissile bonds by tRNA. Because tRNA is approximately twice as large as ProTα itself, such a steric hindrance should be expected and indeed vigorous trypsin treatment of the tRNA-peptide E was required to convert it into tRNA-peptides A_C, B_C and D. The peptide moieties of the tRNA-peptides may be assigned as follows (see also Fig. 5 for schematic representation of the data). E results from trypsin cleavage at Lys-88 or Arg-89 (Fig. 1A) and covers the carboxy-terminal region of ProTα up to the $(His)_6$ tag. D originates from the cleavage within the basic block 102–106 and includes the tag. The untagged peptide B_C results from the cleavage within the 88–89 and 102–106 basic stretches. Thus, the observed pattern of tRNA-peptides is consistent with tRNA attachment within the basic block 102–106. However, if this carboxy-terminal tRNA attachment site is not unique and an additional site occurs upstream from the basic cluster 102–106, then, B_C may also correspond to a tRNA-linked peptide flanked by Arg-89 and Lys-102, as shown in Fig. 5. To test this possibility, we performed tryptic digestion of the tRNA-linked mutant II lacking the basic region 102–106 and analyzed the resultant tRNA-peptides by means of Ni-NTA affinity chromatography and gel electrophoresis (Fig. 3H). It became evident that the second carboxy-terminal tRNA attachment site exists, as manifested by the $(His)_6$-tagged (Ni-NTA bound) short tRNA-peptide. Two short tRNA-peptides in the unbound fraction (Fig. 3H) evidently originate from the amino-terminal portion of ProTα. We conclude therefore that the second tRNA attachment site occurs within the 89–98 region of ProTα.

3.3. The tRNA linking capacity of the central portion of ProTα can be restored by the addition of just a few amino acid residues

To test whether our assignment of tRNA linking sites to the terminal regions of ProTα is correct, we constructed a ProTα mutant V (residues 32–83, Fig. 1B) lacking both of these regions and encompassing the central highly acidic portion of the ProTα molecule and tested it for its ability to link tRNA. Although this recombinant protein could be detected in cell extracts, no complex formation was observed (Fig. 4, lane 1). Carboxy-terminal extension of this protein to form mutant VI (residues 32–101, Fig. 1B) restored the tRNA linking capacity of the ProTα fragment (Fig. 4, lane 2). This result was predictable because the added region included an already identified tRNA linking region, residues 89–98. More surprisingly, the amino-terminal extension of mutant V by three residues only (Gly/Arg/Asp) resulting in mutant VII (residues 29–83) could rescue the tRNA linking capacity of this ProTα mutant as well (Fig. 4, lane 3). Although this result does not

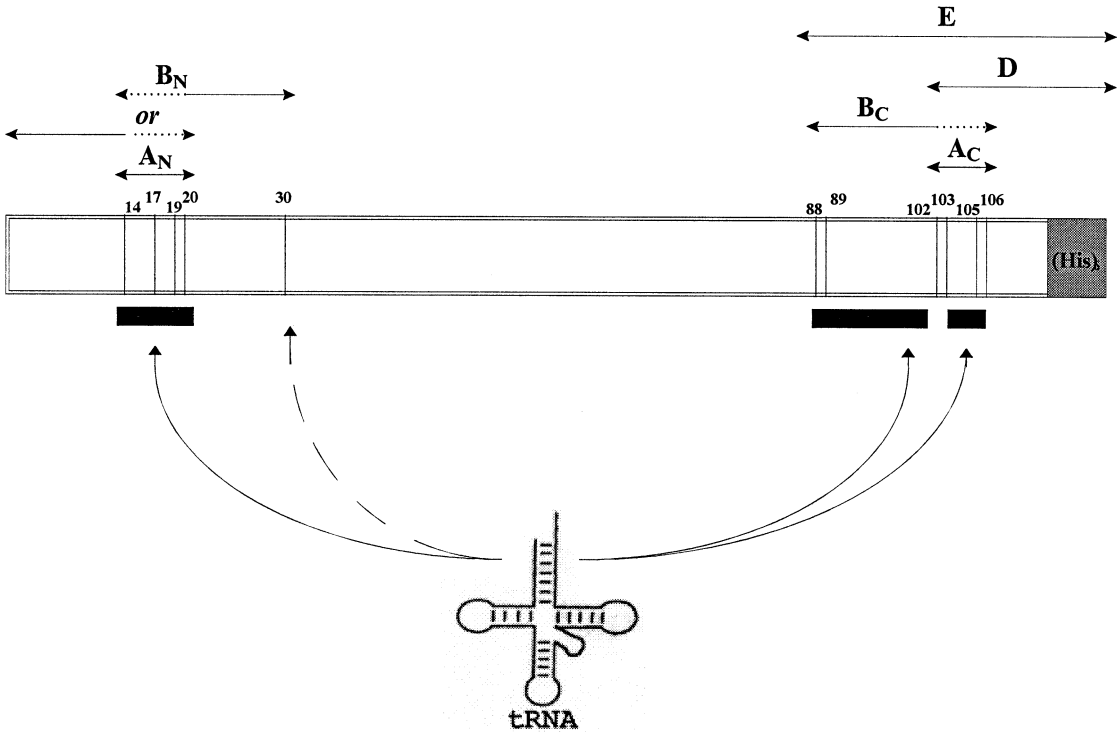


Fig. 5. Schematic representation of tRNA attachment sites in ProTα. The ProTα molecule is depicted by a thick empty rectangle. Vertical bars with numbering above indicate the positions of basic residues in ProTα used to map tRNA attachment sites. Positions of tRNA-linked peptides A, B, C, D and E are shown above ProTα with the bidirectional lines. Dotted lines indicate ambiguities in the boundaries. Solid rectangles below ProTα pointed by the thick arrows show the regions of tRNA attachment. A dashed arrow points to a three amino acid long region restoring tRNA linking to the central acidic portion of ProTα. The tRNA image is reduced. If drawn to scale, tRNA should be approximately twice as large as ProTα. For details, see text.

prove that tRNA attachment occurs to one of the amino acid residues added, it points to an important role of this region in formation of the complex.

4. Discussion

We applied deletion mutagenesis analysis to ProTα, a nuclear mammalian protein involved in cell proliferation [12–18], in order to locate tRNA attachment sites within its molecule. The approach we used is based on a recently observed phenomenon that recombinant mammalian ProTα expressed in *E. coli* cells retains its ability to link a RNA [7], in accordance with its ability to do so in mammalian cells [5,6]. This approach has already been proven to be successful and has enabled us to identify a broad range of bacterial tRNAs as components of the ProTα-RNA complex [7]. Here, we report a surprising observation that there are several tRNA attachment sites in ProTα located close to both termini of the protein. We identified two such sites in the carboxy-terminal region of ProTα and at least one site within the amino-terminal region (Fig. 5). The central highly acidic portion of ProTα appeared to be incompetent in tRNA linking, unless it was extended towards one or both termini of the molecule. To exclude a possibility that multiple tRNA attachment sites arose due to deletion mutagenesis of ProTα, we also employed a complementary approach including site-specific fragmentation of the ProTα moiety of the complex with an endoproteinase, whose cleavage site was introduced in ProTα. The results of this experiment confirmed the presence of several tRNA attachment sites in full-length ProTα and, moreover, suggested that the amino-terminal site(s) may be utilized for tRNA linking more frequently than the carboxy-terminal sites.

Attachment of tRNA to ProTα occurs via a stable bond withstanding all rigorous denaturing treatments tested, as well as proteolytic degradation of ProTα, implying the existence of a covalent linkage between the RNA and protein moieties of the complex [7]. The results of the present study appear to emphasize a role of basic amino acids of ProTα in tRNA linking. Judging by the pattern of tRNA-linked peptides, in two instances, tRNA attachment occurs within the clusters of lysine residues located close to both termini of the ProTα molecule. Further, amino-terminal extension of the central highly acidic portion of ProTα, incompetent in tRNA attachment by only three amino acid residues including an arginine, resulted in restoration of tRNA linking by this ProTα derivative. These data, however, do not provide proof that the basic amino acid residues are directly involved in the formation of the linkage. Besides, the results of the previous analysis make the existence of a phosphoamide bond between the components unlikely [7].

Whatever the chemical structure of the linkage could be, the ability of the highly acidic protein to link negatively charged RNA is somewhat surprising. It might be envisaged that this interaction requires bivalent cations and, by inference, that ProTα itself may possess metal binding properties. Although such a property has not been documented for ProTα, it is worth noting that parathymosin, a protein partially similar

to ProTα, is a zinc binding protein [19,20]. It would be therefore of interest to investigate whether the prediction concerning putative metal binding by ProTα is correct.

Judging by the electrophoretic mobilities of the tRNA-linked ProTα and its derivatives in the SDS-containing polyacrylamide gel, one tRNA molecule appears to be attached to one ProTα molecule. Therefore, alternative tRNA linking to one of several available attachment sites occurs. This rises an interesting question whether or not a unique subset of tRNAs is linked to each attachment site, which requires further clarification.

The observed pattern of ProTα modification via tRNA linking is reminiscent to the more common type of protein modification, i.e. multiple and frequently alternative phosphorylation accomplished by protein kinases. Phosphorylation is a powerful way of regulating protein activity and metabolism. A mechanism and possible functional consequences of protein modification via RNA linking are far less clear. Since mammalian ProTα retains its ability to link RNA in bacterial cells, this might be indicative of the autocatalytic activity of ProTα itself or functional groups of both ProTα and tRNA may combine to accomplish this reaction. Previously, when evidence was obtained for a carboxy-terminal tRNA attachment to ProTα, we speculated that RNA linking may result in intermolecular masking of the positively charged bipartite nuclear localization signal of ProTα (residues 88–106) [8] potentially leading to relocalization of ProTα from the nucleus to the cytoplasm [7]. The effect of phosphorylation in close proximity to the nuclear localization signal on the nuclear import of proteins is well-documented [21]. This assumption was also consistent with the earlier observation that, in case of mouse Krebs 2 cells, the ProTα-RNA complex could be isolated from the cytoplasmic fraction [5]. Identification of the second tRNA attachment site overlapping the same region of the nuclear localization signal is consistent with this interpretation as well.

The possibility of a cytoplasmic location of ProTα driven by the covalently attached RNA might be questioned by the observations that (i) ProTα is usually, though not exclusively [22], localized in the nucleus [8,23–25] and (ii) tRNA linking to ProTα appears to be relatively inefficient, at least in the *E. coli* environment, judging by the small proportion of the RNA-linked versus RNA-free ProTα. However, given that ProTα is a highly abundant protein ranging up to 10^7 copies per HeLa cell (A. Evstafieva and A.B.V., unpublished), cytoplasmic localization of less than 1% of ProTα would be below the limits of detection and yet may comprise a very significant cytoplasmic pool of this protein. Besides, the possibility that cytoplasmic localization of ProTα occurs at certain circumstances of the cell life-span only cannot be excluded. Based on the results of our localization of the tRNA attachment sites in ProTα, we hypothesize that a cytoplasmic function of ProTα may exist. A possible role of the amino-terminal tRNA attachment to ProTα is unclear at present.

A choice of ProTα modification via RNA linking, in addition to phosphorylation which has also been described for ProTα [26–28], is notable. A likely explanation for this phenomenon would be the participation of ProTα in some aspect of tRNA metabolism. Thus, further studies are required to elucidate the role of the ProTα-tRNA complex in the functioning of both of its constituents.

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References

[1] Wimmer, E. (1982) Cell 28, 199–201.
[2] Vartapetian, A.B. and Bogdanov, A.A. (1987) Prog. Nucleic Acid Res. Mol. Biol. 34, 209–251.
[3] Paul, A.V., van Boom, J.H., Filippov, D. and Wimmer, E. (1998) Nature 393, 280–284.
[4] Fontoura, B.M.A., Sorokina, E.A., David, E. and Carroll, R.B. (1992) Mol. Cell. Biol. 12, 5145–5151.
[5] Vartapetian, A.B., Makarova, T.N., Koonin, E.V., Agol, V.I. and Bogdanov, A.A. (1988) FEBS Lett. 232, 35–38.
[6] Makarova, T., Grebenshikov, N., Egorov, C., Vartapetian, A. and Bogdanov, A. (1989) FEBS Lett. 257, 247–250.
[7] Vartapetian, A., Lukashev, D., Lyakhov, I., Belyaeva, A., Chichkova, N. and Bogdanov, A. (1997) RNA 3, 1173–1181.
[8] Rubtsov, Y.P., Zolotukhin, A.S., Vorobjev, I.A., Chichkova, N.V., Pavlov, N.A., Karger, E.M., Evstafieva, A.G., Felber, B.K. and Vartapetian, A.B. (1997) FEBS Lett. 413, 135–141.
[9] Frangou-Lazaridis, M., Clinton, M., Goodall, G.J. and Horecker, B.L. (1988) Arch. Biochem. Biophys. 263, 305–310.
[10] Sburlati, A.R., Manrow, R.E. and Berger, S.L. (1990) Protein Exp. Purif. 1, 184–190.
[11] Evstafieva, A.G., Chichkova, N.V., Makarova, T.N., Vartapetian, A.B., Vasilenko, A.V., Abramov, V.M. and Bogdanov, A.A. (1995) Eur. J. Biochem. 231, 639–643.
[12] Eschenfeldt, W.H. and Berger, S.L. (1986) Proc. Natl. Acad. Sci. USA 83, 9403–9407.
[13] Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J.G., Bustelo, X.R. and Freire, M. (1989) J. Biol. Chem. 264, 8451–8454.
[14] Conteas, C.N., Mutchnick, M.G., Palmer, K.C., Weller, F.E., Luk, G.D., Naylor, P.H., Erdos, M.R., Goldstein, A.L., Panneerselvam, C. and Horecker, B.L. (1990) Proc. Natl. Acad. Sci. USA 87, 3269–3273.
[15] Sburlati, A.R., Manrow, R.E. and Berger, S.L. (1991) Proc. Natl. Acad. Sci. USA 88, 253–257.
[16] Eilers, M., Schirm, S. and Bishop, J.M. (1991) EMBO J. 10, 133–141.
[17] Wu, C.-L., Shiau, A.-L. and Lin, C.-S. (1997) Life Sci. 61, 2091–2101.
[18] Rodriguez, P., Vinuela, J.E., Alvarez-Fernandez, L., Buceta, M., Vidal, A., Dominguez, F. and Gomez-Marquez, J. (1998) Biochem. J. 331, 753–761.
[19] Brand, I.A., Heinickel, A., Kratzin, H. and Soling, H.-D. (1988) Eur. J. Biochem. 177, 561–568.
[20] Trompeter, H.-I., Brand, I.A. and Soling, H.-D. (1989) FEBS Lett. 253, 63–66.
[21] Jans, D.A. (1995) Biochem. J. 311, 705–716.
[22] Tsitsiloni, O.E., Yialouris, P.P., Sekeri-Pataryas, K. and Haritos, A.A. (1989) Experientia 45, 332–334.
[23] Watts, J.D., Cary, P.D., Sautiere, P. and Crane-Robinson, C. (1990) Eur. J. Biochem. 192, 643–651.
[24] Clinton, M., Graeve, L., El-Dorry, H., Rodriguez-Boulan, E. and Horecker, B.L. (1991) Proc. Natl. Acad. Sci. USA 88, 6608–6612.
[25] Manrow, R.E., Sburlati, A.R., Hanover, J.A. and Berger, S.L. (1991) J. Biol. Chem. 266, 3916–3924.
[26] Barcia, M.G., Castro, J.M., Jullien, C.D. and Freire, M. (1993) J. Biol. Chem. 268, 4704–4708.
[27] Perez-Estevez, A., Diaz-Jullien, C., Covelo, G., Salgueiro, M.T. and Freire, M. (1997) J. Biol. Chem. 272, 10506–10513.
[28] Trumbore, M.W., Wang, R.-H., Enkemann, S.A. and Berger, S.L. (1997) J. Biol. Chem. 272, 26394–26404.