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Evidence for aminoacylation-induced conformational changes in human mitochondrial tRNAs

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ABSTRACT **Analysis by acid polyacrylamide/urea gel electrophoresis of 14 individual mitochondrial tRNAs (mt-tRNAs) from human cells has revealed a variable decrease in mobility of the aminoacylated relative to the nonacylated form, with the degree of separation of the two forms not being correlated with the mass, polar character, or charge of the amino acid. Separation of the charged and uncharged species has been found to be independent of tRNA denaturation, being observed also in the absence of urea. In another approach, electrophoresis through a perpendicular denaturing gradient gel of several individual mt-tRNAs has shown a progressive unfolding of the tRNA with increasing denaturant concentration, which is consistent with an initial disruption of tertiary interactions, followed by the sequential melting of the four stems of the cloverleaf structure. A detailed analysis of the unfolding process of charged and uncharged $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ has revealed that the separation of the two forms of these tRNAs persisted throughout the almost entire range of denaturant concentrations used and was lost upon denaturation of the last helical domain(s), which most likely included the amino acid acceptor stem. These observations strongly suggest that the electrophoretic retardation of the charged species reflects an aminoacylation-induced conformational change of the 3′-end of these mt-tRNAs, with possible significant implications in connection with the known role of the acceptor end in tRNA interactions with the ribosomal peptidyl transferase center and the elongation factor Tu.**

The importance of the secondary and tertiary structure of tRNAs for their interactions with aminoacyl-tRNA synthetases, ribosomes, initiation and elongation factors and other proteins, and some of the structural changes caused in the tRNAs by these interactions have been well documented (1–6). Despite the numerous studies in this area (7–15), open questions have remained as to whether aminoacylation of tRNAs modifies their conformation and to what functional role any such modification may have. In a recent analysis of the *in vivo* aminoacylation capacity of some wild-type and mutant human mitochondrial (mt)-tRNAs carried out by the use of electrophoresis in an acid polyacrylamide/urea gel (APUGE) system to separate an uncharged tRNA species from the corresponding charged tRNA, a variability among different tRNAs was observed in the capacity of this electrophoretic system to resolve the aminoacylated and nonaminoacylated forms. This technique had been previously utilized for the study of *in vivo* aminoacylation of several eukaryotic (16) and prokaryotic (17) tRNA species, but the mechanism(s) underlying the separation of the charged and uncharged tRNA species was not investigated.

In the present work, to understand the basis for the effectiveness of this technique in resolving the aminoacylated and nonaminoacylated forms of tRNAs, the analysis of *in vivo* aminoacylation by APUGE has been extended to a broad range of mt-tRNAs (14 out of 22) specific for 13 different amino acids, and complemented by a novel application of denaturing gradient gel electrophoresis (DGGE). This work has unexpectedly provided evidence for the occurrence of aminoacylation-dependent conformational changes of tRNAs.

MATERIALS AND METHODS

Cell Lines and Media. The human cell line 143B.TK$^-$ (18), was grown in Dulbecco's modified Eagle's medium with 5% fetal calf serum and 100 μg of bromodeoxyuridine per ml. HeLa S3 cells were grown in suspension as described previously (19).

Purification of the tRNAs. Highly purified mt-RNA preparations were obtained by phenol-chloroform extraction of nucleic acids from twice EDTA-washed mitochondria of 143B.TK$^-$ or HeLa cells (2–3 × 10^8 and ≈3.0 × 10^9 cells, respectively), followed by sucrose gradient fractionation and polyacrylamide gel electrophoresis, as described previously (20, 21). Cytoplasmic tRNAs from HeLa cells were purified under the same conditions from the postmicrosomal supernatant.

In the experiments in which aminoacyl-tRNA complexes had to be preserved, a much more rapid procedure for the isolation of the mitochondrial fraction (21) and a method of RNA extraction under acid conditions (22, 23) were used.

Electrophoretic Analysis of tRNAs. The highly purified tRNA preparations were electrophoresed through a 15% polyacrylamide/7 M urea gel in Tris borate·EDTA buffer (TBE) and then electroblotted onto a Zeta-Probe membrane (Bio-Rad) for sequential hybridization analysis with specific tRNA probes (see below). In the experiments in which the aminoacylated tRNAs synthesized *in vitro* or in isolated organelles or pre-existing *in vivo* (see below) had to be analyzed, the tRNA fraction or total mt-RNA isolated under acid conditions was electrophoresed at 4°C through a 6.5% polyacrylamide/7 M urea gel in 0.1 M sodium acetate (pH 5.0 or 6.0, as specified below), at 100–200 V (21). The gels were then either treated with Amplify (Amersham), dried and exposed for fluorography, or electroblotted onto a Zeta-Probe membrane for sequential hybridization with specific tRNA probes, as detailed below.

For DGGE, samples of total mitochondrial RNA purified under acid conditions from 143B.TK$^-$ cells were electrophoresed at 4°C through an acid (pH 5.0) 10% polyacrylamide gel containing a denaturing urea gradient (0–8 M) perpendicular to the direction of the electrophoretic run (26), then electroblotted onto a Zeta-Probe membrane and hybridized sequentially with specific tRNA probes (see below).

Identification of mt-tRNAs and Quantification of Charged and Uncharged Forms. Identification of individual mt-tRNAs and determination of the relative amounts of the charged and uncharged tRNA species in mitochondria were made by sequential hybridization analysis of RNA blots with specific probes, using either the mt-tRNA fraction or total mt-RNA isolated and electrophoresed under either acid or nonacid conditions, as described above. As probes for the detection of the different tRNAs, ^{32}P-5′-end-labeled oligodeoxynucleotides were used (Ta-

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Abbreviations: mt-tRNA: mitochondrial tRNA; APUGE: acid polyacrylamide/urea gel electrophoresis; DGGE: denaturing gradient gel electrophoresis.

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ble 1). Most oligodeoxynucleotides were complementary to the 3′-end-proximal region of the acceptor stem and to the whole TψC-loop of a given tRNA. In some cases, the deoxynucleotides complementary to the -CCA end were also included (Table 1). The hybridization reaction with each oligodeoxynucleotide probe (2.5–5 pmol, ≈10^7 cpm, per reaction) was carried out in 15–20 ml of a medium containing 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM trisodium citrate)/50% formamide/5 × Denhardt's solution/0.1% SDS/200 μg of salmon sperm DNA per ml, for 1 h at 37°C. After hybridization, the blot was washed two times for 10 min in 2 × SSC/0.1% SDS at 37°C. Quantification of radioactivity in each band in the blot was made by PhosphorImage detection and ImageQuant analysis (Molecular Dynamics). The blot was stripped according to a standard procedure, and tested for residual radioactivity before the subsequent hybridization reaction.

***In Vitro* and *in Organello* Aminoacylation Assays.** *In vitro* aminoacylation of cytoplasmic tRNAs was carried out as described previously (21, 23), using a crude aminoacyl-tRNA synthetase fraction prepared from HeLa cell mitochondria. The aminoacylation reaction was performed in 50 μl using ≈1 μg of purified tRNA, ≈25 μg protein of the aminoacyl-tRNA synthetase preparation and 75 μCi of [^{3}H]lysine (77 Ci/mmol) (Amersham). After 30 min at 37°C, the aminoacylated tRNA was purified under acid conditions, and ethanol precipitated three times. The reaction products were analyzed by electrophoresis under acid conditions, as described above.

For *in organello* aminoacylation, mitochondria were purified by the rapid procedure (21), and the mitochondrial pellets were then resuspended in the incubation buffer and spun down at 13,000 × *g* for 1 min in an Eppendorf microcentrifuge. Incubation of the isolated mitochondria was carried out under conditions described in refs. 24 and 25 for *in organello* RNA and DNA synthesis, except that the medium contained a mixture of all amino acids (except the labeled one) at a final concentration of 10 μM each, and, instead of the labeled nucleic acid precursors, 75 μCi of either [^{3}H]lysine (77 Ci/mmol) or [^{3}H]leucine (77 Ci/mmol) (21). After 15-min incubation at 37°C, analysis of aminoacylation was carried out as described above.

RESULTS

Identification of Different mt-tRNAs by Hybridization with Specific Oligodeoxynucleotides. For the identification of the different species of mt-tRNA by APUGE, a set of oligodeoxynucleotides, 19–24 nucleotides in length, were synthesized (Table 1). To test the specificity of the oligodeoxynucleotide probes, equal amounts (1 μg) of cytosolic or mt-tRNAs, purified from the postmicrosomal or the mitochondrial fraction of HeLa cells, respectively, were run through a 15% polyacrylamide/7 M urea gel in TBE, electroblotted onto a nylon membrane, and hybridized sequentially with each of the oligodeoxynucleotides ^{32}P-5′-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. As expected from the known average difference in size between cytosolic and mt-tRNAs (27), the ethidium bromide-stained gel revealed that, under the electrophoretic conditions used here, cytosolic tRNAs had an overall lower electrophoretic mobility than mt-tRNAs (not shown).

After hybridization with the specific probes, a single band was found to be labeled by each oligodeoxynucleotide in the portion of the blot containing the mt-tRNA fraction (not shown). Each band had, in general, an electrophoretic mobility corresponding to the size of the mt-tRNA expected to be recognized by that probe, as determined from the Cambridge sequence (28). In particular, among the tRNAs analyzed, only $tRNA^{Met}$ exhibited a significantly lower and $tRNA^{Phe}$ a slightly lower than expected electrophoretic mobility, for unknown reasons. No hybridization signal was detected with any mt-tRNA-specific probe in the portion of the blot containing the cytosolic tRNAs (not shown). From the experiments described above, one can conclude that, under the hybridization conditions used here, no oligodeoxynucleotide probe specific for a mt-tRNA showed crosshybridization with either other mt-tRNAs or cytosolic tRNAs.

Separation of Charged and Uncharged Forms of mt-tRNAs by APUGE. Fig. 1*A* shows a representative experiment in which total mitochondrial nucleic acids were purified under acid conditions from the 143B.TK$^-$ human cell line, electrophoresed through an acid (pH 5.0) 6.5% polyacrylamide/7 M urea gel at 4°C (22), and subsequently electroblotted onto a nylon membrane. After sequential hybridization of the same blot with specific probes, in all cases, but four ($tRNA^{Glu}$, $tRNA^{Asp}$, $tRNA^{Met}$, and $tRNA^{Phe}$), two bands, of which the slower moving was the predominant one, were observed. A similar separation of charged and uncharged tRNA species was obtained for $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Leu(UUR)}$, and $tRNA^{Leu(CUN)}$ by electrophoresis through a 6.5% polyacrylamide/7 M urea gel at pH 6.0 (not shown). The identification of the lower band as uncharged tRNA was made by running in parallel a sample of mt-tRNA deacylated by heating for 5 min at 75°C at pH 8.3. As shown previously for mt-$tRNA^{Lys}$ (22) and as is illustrated here for $tRNA^{Val}$ and $tRNA^{Ser(AGY)}$ (Fig. 1*B*), the deacylated sample gave in all cases a single hybridization band comigrating with the lower of the two labeled bands in the acid-purified tRNA sample. Further evidence that the upper band represented the charged tRNA species was provided by the experiment shown in Fig. 1*C*, in which total HeLa cell mt-tRNA, labeled in isolated organelles with [^{3}H]leucine and extracted under acid conditions (21), was electroblotted onto a nylon membrane and probed with a specific $tRNA^{Leu(UUR)}$ or $tRNA^{Leu(CUN)}$ oligodeoxynucleotide probe. One can see that, for both tRNA species, the band labeled *in organello* with [^{3}H]leucine was the upper one.

Table 1. tRNA-specific oligodeoxynucleotide probes

tRNA	Oligodeoxynucleotide sequence*	Size (mer)
Trp	5′-TGG CAG AAA TTA AGT ATT GC-3′	(20)
Phe	5′-GAG GAG GTA AGC TAC ATA AAC-3′	(21)
Arg	5′-TGG TTG GTA AAT ATG ATT ATC-3′	(21)
Lys	5′-TCA CTG TAA AGA GGT GTT GG-3′	(20)
Met	5′-TAG TAC GGG AAG GGT ATA ACC-3′	(21)
Leu (UUR)	5′-TGT TAA GAA GAG GAA TTG AA-3′	(20)
Leu (CUN)	5′-TAC TTT TAT TTG GAG TTG CA-3′	(20)
His	5′-GGT AAA TAA GGG GTC GTA AGC-3′	(21)
Gly	5′-TAC TCT TTT TTG AAT GTT G-3′	(19)
Glu	5′-TAT TCT CGC ACG GAC TAC AA-3′	(20)
Val	5′-GTC AGA GCG GTC AAG TTA AG-3′	(20)
Ile	5′-TAG AAA TAA GGG GGT TTA AGC-3′	(21)
Asp	5′-GTA AGA TAT ATA GGA TTT AG-3′	(20)
Ser (AGY)	5′-TTA GCA GTT CTT GTG AGC TTT CTC-3′	(24)

*The underlined nucleotides correspond to the region complementary to the 3′-CCA end of the tRNA.

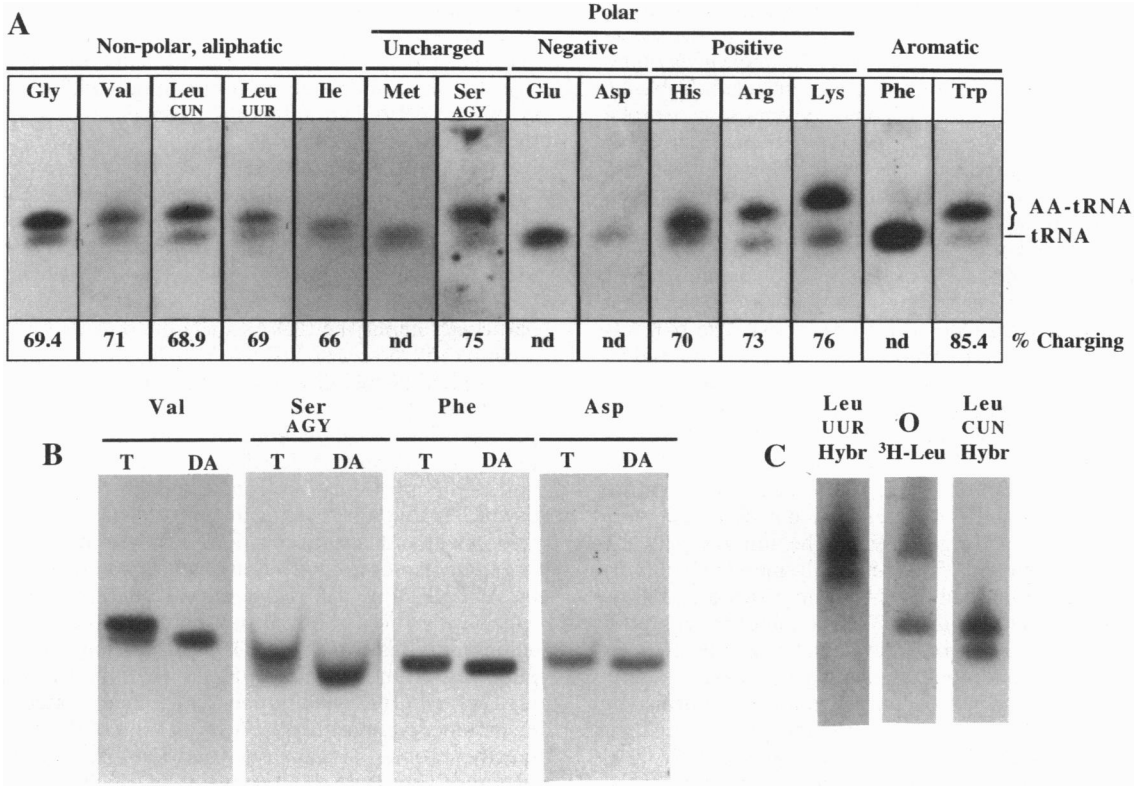

FIG. 1. (*A*) Separation between the charged and uncharged forms of different tRNAs and quantification of the *in vivo* proportion of aminoacylation. Samples of total mitochondrial nucleic acids purified under acid conditions from 143B.TK$^-$ cells (21) were electrophoresed at 4°C through an acid 6.5% polyacrylamide/7 M urea gel (pH 5.0); the conditions used were as described elsewhere (21). The gels were then electroblotted onto a Zeta-Probe membrane, and this was incubated sequentially with ^{32}P-5′-end-labeled oligodeoxynucleotide probes specific for the indicated mt-tRNAs. The percentages of *in vivo* aminoacylation of the various tRNAs are indicated below each lane; nd: not determined. (*B*) Equivalent samples of total RNA mitochondrial purified under acid conditions from 143B.TK$^-$ cells (T) were electrophoresed under the conditions described above, in parallel with samples deacylated by heating for 5min at 75°C at alkaline pH (8.3) (DA), electroblotted, and hybridized sequentially with the indicated mt-tRNA probes. (*C*) Samples of *in organello* [^{3}H]leucine-labeled mt-tRNAs (21) (O) were electrophoresed under acid conditions, electroblotted onto a Zeta-Probe membrane, and hybridized sequentially with the indicated mt-tRNA probes. T, tRNA purified under acid conditions; DA, deacylated tRNA; O, *in organello* aminoacylated tRNA.

From the experiments described above, one can conclude that, for 10 of the 14 mt-tRNAs tested, the presence of the covalently bound amino acid produced a decrease in electrophoretic mobility of the tRNA large enough to resolve it from the uncharged species. However, also in the four exceptions, the covalently bound amino acid did produce a slight gel retardation, as determined by comparing the migration of the charged tRNA with that of the fully deacylated tRNA run in an adjacent lane; however, in these cases, the difference in mobility was not such as to resolve the two forms of tRNA (Fig. 1*B*). As shown in Fig. 1*A*, the proportion of aminoacylation of all the mt-tRNAs analyzed that gave a good resolution of the charged and uncharged species varied within a narrow range, i.e., between 66% for tRNA^{Ile} and 85.4% for tRNA^{Trp}, with an average value of 72.4%.

As concerns the mechanism whereby the covalently bound amino acid reduces the electrophoretic mobility of the corresponding tRNA no univocal correlation could be established with the physicochemical properties of the amino acid. Thus, it had been previously suggested (16, 22) that the positive charge of lysine contributed to the reduction in electrophoretic mobility of lysyl-tRNA^{Lys}. In the experiments described above, this charged tRNA exhibited indeed the highest retardation among the acyl-tRNAs tested. However, in this case, the positive charge could not be the only, nor probably the main factor involved. In fact, arginyl-tRNA exhibited a lower reduction in electrophoretic mobility than lysyl-tRNA (Fig. 1*A*), although the pK for arginine (12.5) is higher than that of lysine (10.5). Moreover, one would have expected that, if the charge of the amino acid played a determining role in the phenomenon, assuming a minor compensating effect of the increase in mass associated with the amino acid (see below), the negatively charged glutamic acid and aspartic acid would have increased the mobility of the acylated-tRNA, and this was clearly not the case (Fig. 1*A*). Furthermore, the results of Fig. 1*A* indicated that it is possible to obtain a very clear separation between the acylated and non-acylated forms of a tRNA regardless of the polar character or charge of the amino acid. In addition, the observation that glycine, the smallest and a nonpolar amino acid, did produce a good separation between the two forms of tRNA, while the much larger phenylalanine did not, and, likewise, the significant difference in extent of separation of the charged and uncharged tRNA produced by isoleucine and leucine (CUN), which have an identical mass, excluded any significant role in the phenomenon of the size of the amino acid.

The results described above suggested the possibility that the covalently bound amino acid produces a conformational change in the tRNA that reduces the electrophoretic mobility of the charged species in APUGE. This possibility was supported by the results of an experiment in which raising the temperature of the APUGE run from 4°C to 25°C drastically reduced the mobility of the charged mt-tRNA^{Lys}, labeled in isolated organelles with [^{3}H]lysine, relative to that of *in vitro* aminoacylated cytosolic tRNA^{Lys}s, strongly suggesting that the tRNAs were not completely unfolded in 7 M urea at 4°C (Fig. 2). Very significantly, the separation of the charged and uncharged mt-tRNA^{Lys} was lost at 25°C, probably as a result of the complete unfolding of the tRNA (Fig. 2). To investigate the possibility that the proposed aminoacylation-induced conformational changes

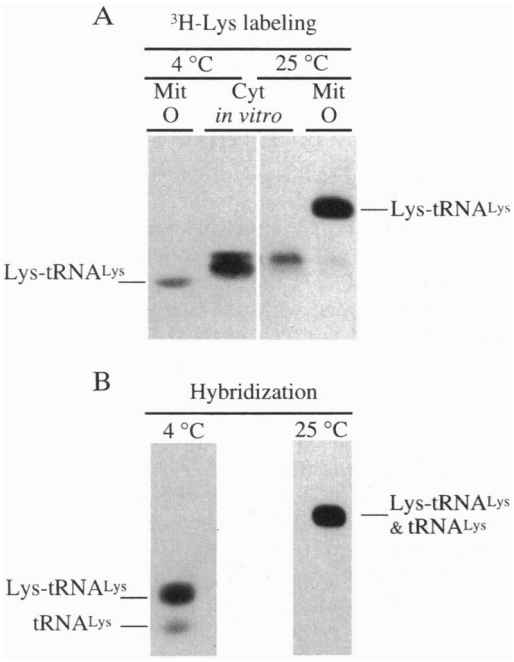

FIG. 2. Influence of the temperature on the electrophoretic mobility of the acylated and nonacylated forms of mt-tRNA^{Lys}. (*A*) Samples of *in organello* [^{3}H]lysine-labeled (O) mt-tRNA (21) were electrophoresed under acid conditions at either 4°C or 25°C, in parallel with *in vitro* [^{3}H]lysine-labeled cytosolic tRNAs (21, 23). (*B*) Equivalent samples were electrophoresed under the same conditions, electroblotted onto a Zeta-Probe membrane, and hybridized with a ^{32}P-5′-end-labeled oligodeoxynucleotide probe specific for mt-tRNA^{Lys}. Mit, mitochondrial; Cyt, cytosolic.

of the tRNAs resulted from the denaturing conditions of gel electrophoresis, an analysis was carried out of the separation of charged and uncharged tRNA^{Lys} and tRNA$^{Leu(UUR)}$ by electrophoresis through a 6.5% polyacrylamide gel in 0.1 M Na acetate, pH 5.0. The results revealed that, in this gel system, these aminoacylated and nonaminoacylated tRNA species were more separated (by about 25%) than in an acid polyacrylamide/urea gel (data not shown; see also below).

Sequential Unfolding of mt-tRNAs by Acid Perpendicular DGGE. To investigate further the possibility of an aminoacylation-induced conformational change of the tRNAs, the influence of the tRNA structure on the separation between aminoacylated and nonacylated forms of the tRNA was analyzed by perpendicular DGGE (26). In this type of fractionation, as the molecules of a given tRNA species move through regions across the gel containing increasing concentrations of denaturant (urea), they would be expected to undergo a process of sequential unfolding in a direction perpendicular to the direction of electrophoresis, resulting in a progressively increasing retardation. The profile produced by this retardation, as detected by hybridizing the tRNA molecules of a given species with a specific oligodeoxynucleotide probe, should be comparable to the profile of thermal unfolding of tRNAs, previously investigated by following optical absorbance changes (29, 30) or by a combination of relaxation and proton nuclear magnetic resonance spectroscopy (31).

Fig. 3 shows representative experiments of this type, in which total mt-tRNA isolated under acid conditions was fractionated by acid perpendicular DGGE (with a 0–8 M urea gradient), electroblotted onto a Z-Probe membrane, and hybridized with oligodeoxynucleotide probes specific for tRNA^{Met} (Fig. 3*A*), or tRNA^{Lys} (Fig. 3*B*), or tRNA$^{Leu(UUR)}$ (Fig. 3*C*). A single electrophoretic profile is recognizable in the tRNA^{Met} panel, which corresponds presumably to methionyl-tRNA^{Met}, formyl-methionyl-tRNA^{Met} and uncharged

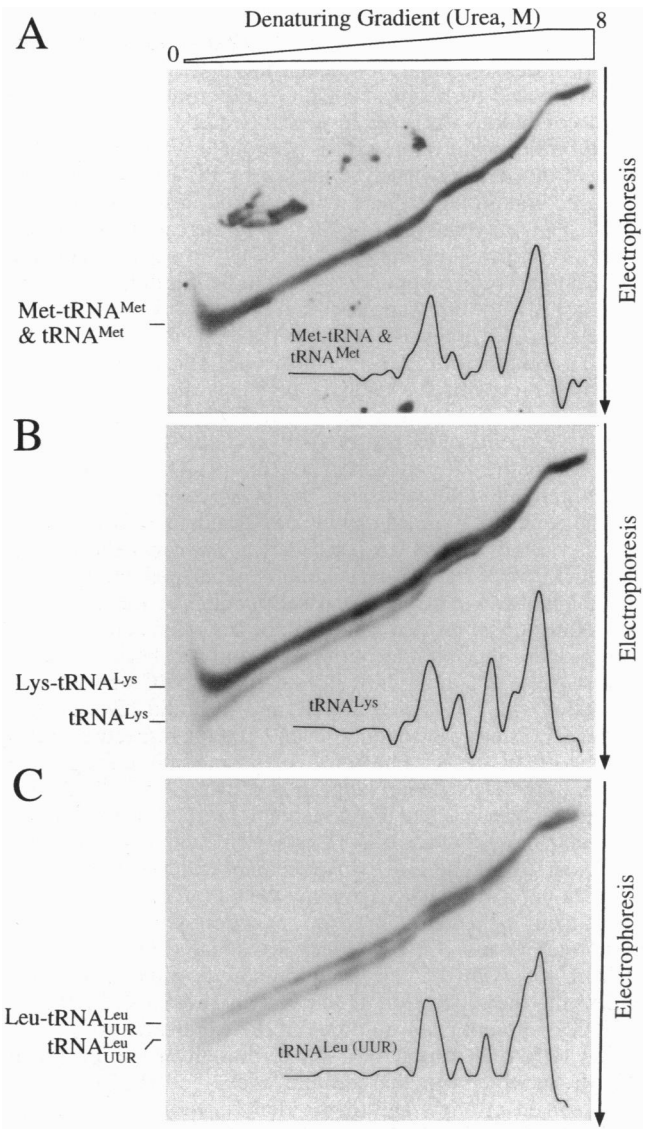

FIG. 3. Influence of the high-order structure of the tRNA on the separation of the acylated and nonacylated forms of several tRNAs. Samples of total mt-RNA purified under acid conditions from 143B.TK$^-$ cells were electrophoresed at 4°C through an acid (pH 5.0) 10% polyacrylamide gel containing a denaturing urea gradient (0–8 M) perpendicular to the direction of the electrophoretic run, then electroblotted onto a Zeta-Probe membrane, and hybridized sequentially (see legend of Fig. 1) with ^{32}P-5′-end-labeled oligodeoxynucleotide probes specific for mt-tRNA^{Met} (*A*), mt-tRNA^{Lys} (*B*) and mt-tRNA$^{Leu(UUR)}$ (*C*).

tRNA^{Met}, whereas the tRNA^{Lys} and the tRNA$^{Leu(UUR)}$ panels exhibit two distinct profiles, which are parallel at the lower urea concentrations and converge at the higher concentrations. Of the latter profiles, the slower moving, predominant one represents presumably the charged tRNA species, and the faster moving one, the uncharged species, in agreement with the results of Fig. 1*A* [the relative over-representation of the uncharged tRNA in the tRNA$^{Leu(UUR)}$, as compared with Fig. 1*A*, is accountable for by the old age of the sample (21)]. As recognizable both in the single profile of tRNA^{Met} and in the double profiles of tRNA^{Lys} and tRNA$^{Leu(UUR)}$, the electrophoretic mobility of each of the three tRNAs decreases progressively across the gel as the molecules move through regions of increasing denaturant concentrations. In particular, in the half of the gel with lower denaturant concentrations, one observes a gradually decreasing mobility of the tRNA, whereas, in the half of the gel with higher denaturant concentrations, the distribution

of the tRNA shows a multiphasic profile, which strikingly resembles the tRNA thermal melting curves (29).

As previously described for the thermal unfolding of tRNA (30, 31), in the acid perpendicular DGGE experiments (Fig. 3), the initial continuous decrease in electrophoretic mobility, as the denaturant concentration increases, probably reflects the relaxation of the tertiary structure caused by the disruption of the hydrogen bonds that stabilize it. The sharp transitions in electrophoretic mobility that occur upon further increase in denaturant concentration are presumably produced by the sequential melting of the domains corresponding to the four helices of the cloverleaf structure. Up to four transitions can be detected in the electrophoretic profiles of mt-tRNA^{Met}, mt-tRNA^{Lys}, mt-tRNA$^{Leu(UUR)}$ (Fig. 3), and other mt-tRNAs (not shown). These transitions are more easily recognized as sharp peaks in the calculated derivative curves of the electrophoretic profiles (Fig. 3). The detailed polyphasic profile of each tRNA is expected to reflect the order of melting of the different helical domains (which depend on their GC content and number of base pairs), as well as the relaxation inducing effect of the each melting event, and, therefore, to differ among various tRNAs. In particular, as concerns the electrophoretic profiles of tRNA^{Lys}, while it is not possible to assign unambiguously the structures corresponding to the first three major transitions, it is very likely that the last prominent transition corresponds to the melting of the acceptor stem, because it is the longest stem and the richest in G–C pairs [3GC and 4AT, as contrasted with 2GC and 3AT, 1GC and 4AT, and 2GC and 2AT for the TΨC stem, anticodon stem and D stem, respectively (28)]. Melting of this helix would produce a randomly coiling single strand. In the electrophoretic profiles of tRNA$^{Leu(UUR)}$, the last prominent transition may correspond to the melting of the acceptor stem (2GC and 5AT) alone or in combination with the TΨC stem (3GC and 2AT) (in agreement with the broadness of the peak in the derivative curve and the partial resolution of two components in this peak), since the anticodon stem (4AT) and the D stem (2GC and 1GT) are much less stable (28).

From the point of view of understanding the mechanism underlying the separation of charged and uncharged tRNA in APUGE, an important outcome of the experiment of Fig. 3*A* is that tRNA^{Met} exhibited a single, though broad, profile in DGGE, in which the charged and uncharged tRNA^{Met} were not resolved at any stage of the denaturation of the tRNA. Similarly, tRNA^{Phe} yielded a single profile through the whole urea concentration gradient in the perpendicular DGGE system (not shown). These results, which fully agreed with those of APUGE analysis (Fig. 1*A*), excluded the possibility that, in the latter fractionation experiments, which were carried out in 7 M urea, the lack of separation of the charged and uncharged tRNA species was due to the molecules being already completely melted in 7 M urea.

In striking contrast to tRNA^{Met} and tRNA^{Phe}, the acylated and nonacylated forms of tRNA^{Lys} and tRNA$^{Leu(UUR)}$ appeared to be separated by perpendicular DGGE, and exhibited a similar profile of retardation in electrophoretic mobility when progressively denatured (Fig. 3 *B* and *C*). Interestingly, the separation between the charged and uncharged forms of tRNA^{Lys} and tRNA$^{Leu(UUR)}$, which was most pronounced at the lowest urea concentrations, approaching the zero level (in confirmation of the electrophoretic runs in acid polyacrylamide gel in the absence of urea, which were mentioned above), was maintained despite the disruption of the tertiary structure of the tRNA. It was reduced, but still evident, after the first three sharp transitions in electrophoretic mobility, but was completely lost with the melting of the last helical domain, which, presumably, included the amino acid acceptor stem. It should be emphasized that the decrease in extent of migration of the tRNA across the gel could not account for the loss of separation of the charged and uncharged forms of tRNA upon melting of the last helical domain. In fact, in an APUGE run in 7 M urea at 4°C, the two forms of tRNA^{Lys} were well separated after 5.5-cm migration (not shown), whereas they were not separated in the DGGE run at 4°C in the 8 M urea region of the gel after 10-cm migration (Fig. 3). Furthermore, as shown above, the two tRNA forms were no longer separated in an APUGE run in 7 M urea at 25°C, after 14.5 cm migration (Fig. 2).

DISCUSSION

The evidence presented in this paper has strongly suggested that aminoacylation induces conformational changes, most probably local, in at least some of the 14 mt-tRNAs analyzed. In particular, two types of observations support this conclusion. (*i*) The degree of retardation in APUGE of the charged species of the various tRNAs was not correlated with the charge, polar character or mass of the amino acid. (*ii*) Analysis of the sequential unfolding of several tRNAs in acid perpendicular DGGE has revealed that the separation of the charged and uncharged species of tRNA persisted throughout the almost entire range of denaturant concentrations used, and was lost at the concentration at which the last helical domain(s) melted.

The most dramatic evidence that the amino acid by itself cannot account for the different mobility in APUGE of charged and uncharged tRNA species is provided by the observation that *Escherichia coli* methionyl-tRNA^{Met} (32) and human cytoplasmic methionyl-tRNA^{Met} (U. RajBhandary, personal communication) have significantly different mobilities from those of the corresponding uncharged tRNAs, whereas human mitochondrial methionyl-tRNA^{Met} has not (present work). These observations clearly show the role of the tRNA sequence in determining the separation of aminoacylated and nonaminoacylated tRNAs in APUGE, strongly supporting the idea of an aminoacylation-induced conformational change. On the other hand, that the amino acid can contribute in some cases, due to charge effect, to the electrophoretic retardation of a charged tRNA is shown by the finding that the same *E. coli* tRNA^{Met} charged with methionine has a faster mobility than when charged with lysine (33).

As to the mechanism underlying the conformational change that is suggested here to contribute in varying degrees to the separation in APUGE of at least some of the charged and the uncharged tRNAs, the evidence presented in this paper excludes the role of denaturing conditions, since an even greater separation was observed for tRNA^{Lys} and tRNA$^{Leu(UUR)}$ in the absence of urea. Therefore, it is a plausible idea that the conformational charge observed in this work reflects a phenomenon that also occurs under native conditions *in vivo*. Within the limits of sensitivity of the perpendicular DGGE approach, the presence of the amino acid did not seem to alter significantly the denaturant concentrations at which the different helical domains melted. This result suggests that the covalently bound amino acid did not influence the base pair interactions of the tRNA in the secondary structure, in agreement with early data indicating that no changes occur in this structure upon aminoacylation (34–36). The observation that, at the final stage of denaturation, there was no longer a separation between the acylated and deacylated forms of tRNA^{Lys} and tRNA$^{Leu(UUR)}$, despite the fact that the contribution of the amino acid, as well as of any possible aminoacylation-related secondary modification of the tRNA, to the overall mass and charge of the molecule remained unaltered relative to the earlier stages of tRNA unfolding, rules out a major role of these factors in the electrophoretic retardation of the lysyl-tRNA^{Lys} and leucyl-tRNA$^{Leu(UUR)}$. This result, while fully confirming the conclusions of the APUGE analysis, strongly suggests that the covalently attached amino acid causes gel retardation of the aminoacyl-tRNA by producing a less compact structure relative to the uncharged tRNA. If the interpretation given above of the melting behavior of tRNA^{Lys} and tRNA$^{Leu(UUR)}$ is correct, the proposed aminoacylation-induced relaxation of these tRNAs would appear to be restricted to, or at least to affect predominantly the amino acid acceptor domain. The observation that all the tRNAs analyzed exhibited, although

to varying degrees, a reduced electrophoretic mobility of the aminoacylated relative to the uncharged form suggests that, under the conditions used in the present experiments, this loss of compactness of the tRNA structure may be a general characteristic of the aminoacylated tRNAs.

Previous work aimed at investigating by a variety of physical techniques (7–15) the occurrence of conformational changes of tRNAs upon aminoacylation had given conflicting results, leaving the question open. It should be emphasized that the perpendicular DGGE method, because of its extreme sensitivity (26), the possibility of analyzing independently and at the same time the acylated and nonacylated forms of most tRNA species, without the occurrence of a significant interconversion between them, and without previous purification (17), and because the tRNAs in this method are embedded in a gel matrix under a low constant voltage, that can almost "freeze" the molecules in their most favored conformation, offers distinct advantages over previously used techniques.

The evidence discussed above that points to the 3′-end of $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ as the predominant site of the proposed conformational change is particularly significant in view of the essential role of the 3′-aminoacylated-CCA in the proper functional interactions between tRNA and the peptidyl tranferase center of the ribosome (37) and elongation factor Tu (6). Recent evidence from enzymatic (38), chemical reactivity (2), nuclear magnetic resonance (39), and crystallographic studies (4–6, 40–42) has indicated that the conformation at the 3′-end can vary among tRNAs depending on the identity of the discriminatory base and neighboring base pair of the acceptor stem, and, in the same tRNA, when it interacts with proteins. In particular, it has been shown that the 3′-end of the tRNA can exist in either an extended configuration continuing the stacking of the acceptor stem or at least two fold-back configurations (39). In both fold-back conformations, an interaction of unpaired nucleotides of the 3′-end with base pairs of the acceptor stem has been detected (39). These data suggest that the conformation of the 3′-end terminus of the acceptor stem is flexible (1). Furthermore, it has been suggested that the 3′-end conformation can in turn affect the interaction of tRNAs with proteins (2, 39). It is, therefore, a plausible hypothesis that the conformation of the acceptor stem and 3′-end of the tRNA could become more "relaxed" upon aminoacylation, in such a way as to favor the interactions of the tRNA with the peptidyl transferase P site (37), elongation factor Tu (6), and, possibly, peptidyl-tRNA hydrolase (39). It is conceivable that one important factor contributing to the varying degree of aminoacylation-induced retardation of different tRNAs in APUGE is whether the 3′-end of the uncharged tRNA is in extended stacked or folded back conformation.

In a different context, another interesting conclusion of the experiments reported here is the high level of *in vivo* aminoacylation of human mt-tRNAs. The small differences observed among different tRNAs in the proportion of charged species did not correlate with the relative abundance of the tRNAs in mitochondria of HeLa cells (20), nor with the corresponding relative codon usage in translation (28). It should be noted that, in every other wild-type system investigated, no tRNA has been found to exhibit an aminoacylation level lower than 66% (16, 17). Furthermore, it has been shown that, whenever the aminoacylation level falls below a certain critical threshold, there is a reduction in elongation rate in protein synthesis (43, 44) or premature translation termination (22). These observations reflect the fact that the balance between charged and uncharged tRNAs plays a crucial role in the translation process, independently of the total level of a given tRNA species (43, 45, 46).

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