Stepping Transfer Messenger RNA through the Ribosome*

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*This work was supported in part by Grant 55000303 from the Howard Hughes Medical Institute, by Grant 02-04-48781 from the Russian Foundation for Basic Research, and Grant 1707.2003.4 from the President of the Russian Federation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a stipendium from the Max Planck Institute for Molecular Genetics (Berlin).

§ Supported by grants from the Swedish Institute and the Swedish Research Council.

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††† The abbreviations used are: tmRNA, transfer messenger RNA; SmpB, small protein B; MS, mass spectrometry; MS/MS, tandem MS; MALDI, matrix-assisted laser desorption ionization; cryo-EM, cryo-electron microscopic; IPTG, isopropyl β-D-1-thio-β-D-galactopyranoside; DIG, digoxigenin; pk, pseudoknot; RF, release factor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]ethylglycine.

The initiation step of the tmRNA (transfer messenger RNA) is a unique molecule used by all bacteria to rescue stalled ribosomes and to mark unfinished peptides with a specific degradation signal. tmRNA is recruited by arrested ribosomes in which it facilitates the translational switch from cellular mRNA to the mRNA part of tmRNA. Small protein B (SmpB) is a key partner for the trans-translation activity of tmRNA both in vivo and in vitro. It was shown that SmpB acts at the initiation step of the trans-translation process by facilitating tmRNA aminoacylation and binding to the ribosome. Little is known about the subsequent steps of trans-translation. Here we demonstrated the first example of an investigation of tmRNA-ribosome complexes at different stages of trans-translation. Our results show that the structural element at the position of tmRNA pseudoknot 3 remains intact during the translation of the mRNA module of tmRNA and that it is localized on the surface of the ribosome. At least one SmpB molecule remains bound to a ribosome-tmRNA complex isolated from the cell when translation is blocked at different positions within the mRNA part of tmRNA.

tmRNA1 (SarA RNA or 10 S RNA (1)) is a small stable RNA that is found in all euubacteria as well as in some chloroplasts and mitochondria (2, 3). Its 3'- and 5'-ends are folded into a tRNA-like structure with an amino acid acceptor stem that possesses identity elements of tRNA4 and enables specific aminoacylation of the tmRNA by alanyl-tRNA synthetase (4, 5). tmRNA has a short open reading frame in the middle of the molecule surrounded by pseudoknots (the mRNA module) that encodes a degradation signal (tag peptide) (6) for certain cellular proteases (ClpXP, ClpAP) (7). The combination of properties for both tRNA and mRNA results in an unusual translational mechanism for this molecule known as “trans-translation,” switching translation from cellular mRNA lacking stop codon to the coding part of tmRNA, thus adding the tag peptide to the truncated polypeptide chain (8).

Several proteins were shown to function during the initiation of tmRNA-mediated trans-translation. Elongation factor Tu is important for initial tmRNA binding to the ribosome (9) and may also facilitate the structural rearrangement of the tmRNA molecule (10). Ribosomal protein S1 may bind to the mRNA part of tmRNA (11) in a manner similar to that of cellular mRNA (12). Small protein B (SmpB) is the only key protein known to be essential and specific for trans-translation. Both in vivo and in vitro studies have shown that trans-translation does not take place in the absence of SmpB (13, 14). This protein also facilitates tmRNA aminoacylation (15). Recently, it was shown that all SmpB molecules are bound to ribosomes within the cell. Moreover, one ribosome can bind two protein molecules that remain bound to the ribosome even after the transfer of undersynthesized peptide to tmRNA (16). SmpB was also found to be the partner of tmRNA on entering the ribosome during the pre-accommodation stage (17). Comparison of the x-ray crystal structure of a ribonucleoprotein complex that includes a tmRNA fragment and SmpB (18) with cryo-electron microscopic (cryo-EM) data for tmRNA-SmpB complex entering the ribosome (17) shows that SmpB protein may change its position within the tmRNA.

The interaction of tmRNA with the ribosome was studied by cryo-EM only at the pre-accommodation stage (17). Little is known about such interactions at later stages of trans-translation. Recently we proposed a hypothetical model describing how tmRNA may first enter and then pass through the ribosome (19). The predicted conformation of tmRNA at the initial stage of its interaction with the ribosome was confirmed by cryo-EM (17). According to our model, several structural elements of tmRNA should remain intact during the passage of tmRNA through the ribosome, and they should be located on the surface of ribosome (19). Here, we have presented the first example of trans-translation study at different stages of the passage of tmRNA through ribosome based on the isolation and investigation of tmRNA-ribosome complexes in which ribosomes stopped at the 4th or 11th codon of the coding part of tmRNA.

EXPERIMENTAL PROCEDURES

Strains—The XL-1 strain of Escherichia coli was used for all of the genetic manipulations. X91 strain (AssrA) (20) was a kind gift of Dr. K. Williams. The SKZ-1 strain of E. coli with disrupted tmRNA gene and thermosensitive RF2 was made and used for the isolation of tmRNA-ribosome complex as follows. The chromosomal prfb + gene of E. coli was replaced by a cotransformed plasmid pRCB18 (21), which contains the ampicillin resistance gene in the BamHI site of pBR322. The original plasmid pRCB18 was then digested with BamHI and EcoRI, and the BamHI-EcoRI fragment containing the ampicillin resistance gene was ligated to the BamHI-EcoRI fragment containing the SsrA RNA gene. The resulting plasmid (pRCB34) was used for transformation of the SKZ-1 strain. The resulting colonies were tested by colony hybridization with an SsrA RNA probe.
coli X91 (20) was replaced with the prfB2 allele by co-transduction with the nearby zpc21:Tap10 marker that gave tetracycline resistance, using phage P1 and UY2687 as a donor strain (21). The transduction was performed according to standard procedures (22). To do that, a plasmid stock was made in UY2687 cells. Strain X91 was then used as a recipient, and selection was made for tetracycline resistance. Co-transduction of the prfB2 allele was determined by screening for decreased ability to grow on low salt LB at 42 °C. The absence of srrA gene in the resulting strain SKZ-1 was confirmed by PCR as described previously (20).

**Plasmids.—**To remove the sequence corresponding to pseudoknot (pk3) and to introduce to the KpnI and SmaI sites (22) to do that, a plasmid stock of streptavidin-binding aptamer (23) carrying the KpnI and SmaI sites in the ends. The resulting plasmid was named pGEM-stra. pGEM-500, cut with KpnI and SmaI, was ligated with annealed oligonucleotides coding for streptavidin-binding aptamer (23) carrying the KpnI and SmaI sites for the subsequent cloning to determine the molar ratio of L2 to SmpB.

**Polyacrylamide gel electrophoresis.—**A protein band at approximately 18–19 kDa (Fig. A and B) from the protein gel was subjected to reduction, alkylation, trypsin digestion, and peptide purification as described previously (27). Desalted peptides were transferred onto a MALDI-MS sample plate, mixed with the MALDI matrix 4-hydroxy-α-cyanoanisol acid, and allowed to dry. Mass measurements were performed on an orthogonal MALDI-triple time-of-flight mass spectrometer, a prOTOF2000 (PerkinElmer Life Sciences/Sciex), within the m/z range from 500 to 10,000. External calibration on the monoisotopic masses of bradykinin fragment 2–9 (m/z 406.4861, Bachem) and pyro-E-neurotensin (m/z 1672.9175, Bachem) resulted in an average mass accuracy below 6.0 ppm for signals of the peptide ions observed. Peptide mass fingerprints were analyzed using Xproteo (www.xproteo.com).

**RESULTS**

### tmRNA Mutants—An aptamer affinity tag was inserted into the tmRNA molecule to allow the isolation of tmRNA-ribosome complexes by affinity chromatography. Earlier we demonstrated that the aptamer to streptavidin was very efficient for the isolation of ribosomes when inserted into 23S rRNA (29). It has been shown that pk3 is absent in the tmRNAs from some eubacteria (2) and that substitution of this pseudoknot in E. coli tmRNA with a single-stranded structure element does not significantly affect the activity of tmRNA (30). We substituted pk3 with the aptamer to streptavidin (Fig. 1). According to our model the streptavidin-binding aptamer is located near the surface of the ribosome (19) pk3 should be located on the surface of the ribosome. Insertion of the aptamer into the pk3 position made the resultantmutant tmRNA 41 nucleotides longer than the wild type. We used the resulting plasmid pGEM-stra to express mutant tmRNA-stra (a tmRNA containing a streptavidin tag and a wild-type tmRNA module) under control of its native promoter and terminator. Mutant tmRNA remained active in the test system (20).

The aptamer-tagged tmRNA mutants were designed in a way to block the translation of the coding region of tmRNA at
specific positions during the termination of the trans-translation process. It is possible to block termination in the UGA stop codon recognized by RF2 if this release factor is inactive. The *E. coli* strain used in this study harbors a temperature-sensitive RF2 mutant that is active at 37 °C and significantly reduces activity at 42 °C (see “Experimental Procedures”). Wild-type tmRNA contains two UAA stop codons recognized by both release factors (RF1 and RF2). We mutated the first UAA stop codon to UGA in tmRNA-stra and changed the sequence around the stop codon (Fig. 1) to one known to be the “weakest termination signal” (31). To catch the complex at earlier stages of the passage of tmRNA through the ribosome we moved the UGA stop codon together with the weakest termination signal to the position of the fourth codon (pGEM-stra-4 expressing the mutant tmRNA-4) (Fig. 1, tmRNA-4). The presence of the UGA stop codon within the weakest termination context together with thermosensitive RF2 dramatically increased the probability of isolating the ribosome complexes containing mutant tmRNAs with the streptavidin tag affinity method.

**Confirming the System**—As designed, the system contains two altered elements: 1) mutant tmRNA with streptavidin aptamer instead of pk3 and a UGA stop codon recognized by RF2 and 2) a thermosensitive RF2 to impair termination at UGA. In the first series of experiments we tested the activity of mutant tmRNAs to determine whether they could tag proteins for subsequent proteolysis. To test the activity of tmRNA mutants we applied the Williams system (20), in which cells became resistant against kanamycin only in the presence of a functioning tmRNA upon IPTG induction of kanamycin resistance gene repressor. Fig. 2A, left panel, shows that all of the cells grew in the absence of IPTG and both mutants, tmRNA-4 and tmRNA-11, as well as without any tmRNA or the presence of wild-type tmRNA. However, only the cells lacking tmRNA were not viable in the presence of IPTG; the cells harboring wild-type or mutant tmRNA were alive (Fig. 2A, right panel). The results clearly show that the mutants tmRNA-4 and -11
The cross-reactivity of the (His6)-antibody.

any tmRNA in the cells thermosensitive RF2. Controls without (w/o) either the strain X91 with a wild-type RF2 or the strain SKZ1 with the mutant RF2 (strain SKZ1). Fig. 2 demonstrates that cross-reacted with the anti-(His6) antibody. This construct was introduced thus allows identification of tagged proteins by commercially available anti-(His6) antibodies. This approach did not allow us to make a quantitative estimation but indicated that most of tRNA in that complex was tRNAPro.

A potential tmRNA-ribosome complex stalled in the initiation stage unable to translate the mRNA module should be the same in the presence of both tmRNA-4 and tmRNA-strata. The fact that we do not observe a tmRNA band in lane 6 (Fig. 3A) indicates that such a potential complex is negligible in the isolated tmRNA-ribosome complex. Scanning the tmRNA bands of Fig. 3A, lanes 2, 4, and 3 (total tmRNA-4 in the S 30 cell-free extract, the tmRNA complexed with the ribosome in the initial ribosomal fraction, and tmRNA in the isolated complex, respectively) and considering the fraction volume applied to the gel revealed that at least 80% of tmRNA from the initial ribosomal fraction or 4% from total (S30 fraction) was bound to the ribosome in the isolated complex.

According to the nucleotide sequence of mutant tmRNAs (tmRNA-11 and tmRNA-4 in Fig. 1) the UGA stop codon should occupy the A site, and the CCA Pro codon should be in the P site. Therefore the tRNA bound to the P site of the ribosome should correspond to tRNAPro. To test this we probed the tRNA in the ribosome tmRNA complex using Northern blot analysis with DIG-labeled DNA specific to tRNAPro. The results of the Northern blot for tRNA from the complexes blocked at either the 4th or the 11th codon are shown in Fig. 3B. The signal clearly demonstrates the presence of tRNAPro in the complex. Rough estimation of the tRNAPro amount was done by comparing the dot-blot signal intensities for tRNAPro from the tmRNA-4 complex with known amounts of T7 tRNAPro transcript (Fig. 3C). This approach did not allow us to make a quantitative estimation but indicated that most of tRNA in that complex was tRNAPro.

Analysis of the Protein Content of tmRNA-Ribosome Complexes—The proteins in the purified complexes containing tmRNA-4 and tmRNA-11 were separated by gel electrophoresis as shown in Fig. 4, A, lane 3, and B, lane 3, respectively. Ribosomal proteins of the 30 S (Fig. 4A, lane TP30) and 50 S subunits (Fig. 4B, lane TP50) served as markers. We used the following controls: (i) proteins from the initial ribosomal fractions of the complexes with tmRNA-4 and tmRNA-11 (lanes 1 in Fig. 4, A and B, respectively) and (ii) proteins derived from flow-through fractions (lanes 2 in Fig. 4, A and B). In the case of wild-type tmRNA no ribosomes were bound to the resin with the exception of at least three proteins from cell extract that were bound to the streptavidin resin. The corresponding bands are marked with asterisks at lanes 3 in Fig. 4, A–C.

Lanes 3 in Fig. 4, A–C, represent the analysis of protein content of the isolated complexes. The protein spectra in Fig. 4, A and B, show that complexes purified via tmRNA-4 and tmRNA-11 contain ribosomal proteins. The bands with the mobility corresponding to elongation factors G, Tu, and Ts (marked by arrows in lane 1 of Fig. 4, A and B) disappear from the blocked complexes (lane 3 in both panels). An additional
Northern blot probed with DIG-labeled DNA oligonucleotide complementing PAGE. The positions of the RNAs are marked with the tag and wild-type stop codon (tmRNA-stra) (column) (1/400), and the complex with tmRNA with the affinity lane 5 through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/200 of the total amount), the initial ribosomal fraction (init. fr.) (see “Experimental Procedures”) (lane 4) (1/400 of the total amount), the flow-through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/400), and the complex with tmRNA with the affinity tag and wild-type stop codon (tmRNA-stra) (1/20 of the total amount), the initial ribosomal fraction (init. fr.) (see “Experimental Procedures”) (lane 4) (1/400 of the total amount), the flow-through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/200 of the total amount), the complex with tmRNA-stra (1/200 of the total amount), the complex with tmRNA-4 (1/20 of the total amount), the flow-through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/200 of the total amount), the complex with wild-type (WT) lane 2 and tmRNA-4 lane 2.

FIG. 3. Analysis of the RNA content of the tmRNA ribosome complexes. A, electrophoretic separation of RNA isolated from the complex with wild-type (WT) tmRNA (lane 1) (1/20 of the total amount), tmRNA-4 isolated from S 30 cell-free extract by affinity chromatography (lane 2) (1/20 of the total amount), the complex with tmRNA-4 (lane 3) (1/20 of the total amount), the initial ribosomal fraction (init. fr.) (see “Experimental Procedures”) (lane 4) (1/400 of the total amount), the flow-through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/400), and the complex with tmRNA with the affinity tag and wild-type stop codon (tmRNA-stra) (1/200 of the total amount), the flow-through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/200 of the total amount), the initial ribosomal fraction (init. fr.) (see “Experimental Procedures”) (lane 4) (1/400 of the total amount), the flow-through fraction (the fraction of the ribosomes that did not bind to the column) (lane 5) (1/200 of the total amount), the complex with wild-type (WT) lane 2 and tmRNA-4 lane 2.

A: tmRNA-4

B: tmRNA-11

C: tmRNA-stra

D: SmpB stoichiometry

SmpB was also present. Likewise, we could not detect SmpB in the same gel position of the control runs with TP30 and TP50 proteins. Thus, we established a characteristic peptide fragmentation signature for the tryptic SmpB peptide ANISDSSVLRR at m/z 1250.67 (detailed in Fig. 5) in the tryptic digest mixtures of the associated bands of A3, TP30, B3, and TP50 (thin arrow lines). The occurrence of the specific fragmentation signal at m/z 750.4 reveals the presence of SmpB. D, estimation of SmpB stoichiometry. Lane 1, complex with tmRNA-11, 50 pmol. Lanes 2 and 4, SmpB-His6 (SmpB*), 50 and 40 pmol, respectively. Lanes 3 and 5, TP70 (proteins isolated from 70 S ribosome), 35 pmol and 25 pmol, respectively. Positions of the proteins L2, SmpB, and SmpB-His6 are marked by arrows. The table shows the scanned band intensities for L2 and SmpB in corresponding lanes.

To estimate the molar ratio of ribosome:SmpB in the com-
Stepping tmRNA through the Ribosome

Fig. 5. MALDI-quadrupole time-of-flight and MALD-Ion trap mass spectrometric analysis of the tryptic peptide mixture derived from a gel band that was suspected to contain SmpB (from the gel depicted in Fig. 4B, lane 3). Monoisotopic masses and their assignment to peptides derived from the proteins S7, SmpB, and trypsin are shown. The inset, a MALDI-ion trap MS/MS spectrum, visualizes how the characteristic peptide fragmentation signature for the SmpB peptide ANISDYSVLLR was obtained. b and y ions, including the loss of water from the parent ions, are labeled. The most intense fragment ion signal was obtained from the y9 ion at m/z 750.38 ± 0.30.

DISCUSSION

Although the process of trans-translation has been studied for almost 10 years, the mechanism of the transition of tmRNA through the ribosome remains mainly unknown because previous studies with in vitro systems revealed only details of the initiation stage. In contrast, our study is based on in vivo systems that were successfully applied to gain mechanistic insights into the passage of tmRNA through the ribosome in trans-translation. tmRNA is a highly structured molecule; in addition to numerous helices, E. coli tmRNA also has four pseudoknots (Fig. 1). Thus the question arises as to whether the ribosome unwinds these structural elements in the course of trans-translation or whether some of these structures are retained. It should be noted that pseudoknot structures are very stable and are not expected to be resolved easily by the ribosome. The pseudoknot presence in mRNA causes translational pausing, a process that can result in translation frameshifting (36). Recently we proposed that tmRNA can pass through the ribosome without destruction of its pseudoknots (19). We suggested that tmRNA should have a domain containing a structure imitating a codon-anticodon duplex that is recognized by the ribosomal A site. This domain should include the tRNA-like region of tmRNA and most probably pk1. The tRNA-like domain should move through the ribosome similar to a canonical tRNA using the same tRNA binding sites on the ribosome. At the same time the other pseudoknots should be located on the 30 S subunit outside of the decoding center. Helix 5, including the mRNA part of tmRNA, should be unwound during trans-translation. Accordingly, the structural rearrangements of tmRNA should take place, and tmRNA could easily leave the ribosome after the termination of trans-translation using the classical termination mechanism.

Recent structural studies of the SmpB complex using a tmRNA fragment that represented the tRNA-like domain showed structural similarity with the tRNA, although the angle between the arms is different in tmRNA (18). SmpB was shown to be necessary for the formation of a proper tmRNA structure recognized by the ribosomal A site. cryo-EM studies of the complex of tmRNA interacting with the ribosome in the pre-initiation stage in which the tRNA-like region of tmRNA remains in the complex with elongation factor Tu-GDP placed pk1 in the decoding center of ribosome. According to the cryo-EM model, another pseudoknot forms an arch-like structure in the 30 S subunit (17). This structure agrees well with our hypothesis. However, the arch-like structure in tmRNA that exists in the pre-initiation complex should undergo conformational changes during later stages of trans-translation. According to our model, after such rearrangement the pseudoknot 3 of tmRNA should be located on the solvent-accessible side of the ribosomal 30 S subunit. If we substitute pseudoknot 3 with another structural element, the aptamer to streptavidin, and if this element remains intact and is localized on the solvent-accessible surface of the 30 S subunit, it should allow binding the tmRNAribosome complex to streptavidin-Sepharose at different steps of trans-translation.

To test our hypothesis it was necessary to prepare such ribosome-tmRNA complexes in which trans-translation would be arrested at different steps of the passage of tmRNA through the ribosome. There are two strategies to obtain such complexes: 1) to use an in vitro system and 2) to stop trans-translation specifically within the cell and isolate the complex. In vitro systems were previously used to study the details of trans-translation only at the initiation stage (17). We decided to take advantage of the ability of the living cell to perform trans-translation very effectively and to isolate complexes of interest from the cell directly. trans-Translation was stopped at the termination step in living cells. Together with the replacement of pk3 for the streptavidin aptamer, the coding region of tmRNA was mutated so that the termination signal could be recognized only by RF2. The codons

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preceeding the stop codon were changed to code for Asp and Pro, which were shown to decrease termination efficiency and provoke ribosome pausing (37). In our experiments these mutant tmRNAs were the only tmRNAs present in a strain that contained a thermosensitive RF2 inactivated at 42 °C. We varied the position of the mutant termination signal within the tmRNA sequence; to test early and later stages of trans-translation, the stop-codon UGA with its context described above was placed into the position of the 4th or 11th codon, respectively. Controls demonstrated that tmRNA-4 and tmRNA-11 were functional and that the isolated complexes did not contain significant amounts of initiation complexes. As a result we obtained two complexes in which tmRNA was tightly bound to the 70 S ribosome. The tmRNAs did not dissociate from the complex even after centrifugation through a sucrose gradient.

Such affinity purification is possible only when the proper structure of the RNA affinity domain is preserved and exposed outside the ribosome. Hence our successful isolation of tmRNAribosome complexes clearly indicates that the structure of the element that we have inserted into the position of pseudoknot 3 of tmRNA remains intact in both tmRNA-4 and tmRNA-11, which represent different stages of tmRNA passage through the ribosome. It follows that the ribosome does not unwind at least the structure of pk3 in the course of trans-translation, implying that this structural element is localized on the ribosome surface, on the solvent side of the 30 S subunit in agreement with cryo-EM data for the pre-initiation complex (17). These observations are in good agreement with our model for the path of tmRNA through the ribosome (19).

The analysis of the RNA content (Fig. 3) showed that RNAs, tRNAPro, and tmRNA were components of the complex and presented in almost equimolar amounts indicating that when a stop codon was in the A site, a tRNAPro was in the neighboring site (P site). Analysis of the protein content for the isolated complexes (Fig. 4) revealed that SmB protein was in both tmRNA-4 and tmRNA-11 complexes.

Our results extend the knowledge about SmB. This protein was found to be crucial for the trans-translation process (13, 14). It has been shown in a number of studies that SmB can bind tmRNA, stimulating its aminoacylation (15). The structure of SmB in the complex with a tmRNA fragment has been solved (18). According to cryo-EM data, SmB is bound to tmRNA in the pre-accommodation complex (17). Recently, it was found that SmB can also be bound to the ribosome (16) and that two molecules of SmB pre-bound to one ribosome facilitate the accommodation of tmRNA in an in vitro system. These data indicate that SmB plays an essential role at the stage of the initiation of trans-translation. We have shown that SmB is also present in tmRNAribosome complexes at the different stages of tmRNA passage through the ribosome. Although we cannot exclude the rebinding of SmB after dissociation from the initiation complex of tmRNAribosome, we can also consider the possibility that SmB may remain bound to the tmRNAribosome complex after the initiation of the trans-translation process until termination. Our finding suggests that the action of SmB may not be exclusively restricted to the initial stages of trans-translation but that it might also be important throughout the overall trans-translation process.

Acknowledgments—We are grateful to Dr. K. P. Williams for providing us with strain X91 and plasmid pSHA and to Y. Teraoka for StrepHis protein. We also thank Prof. J. Dinman and S. V. Kiparisov for helpful discussions.

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