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# Identification of 50S Proteins at the Peptidyl-tRNA Binding Site of *Escherichia coli* Ribosomes

**(protein L2 and L27/affinity labeling/protein synthesis/two-dimensional electrophoresis/antibiotics)**

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**ABSTRACT** **Bromoacetyl-phenylalanyl-tRNA$^{Phe}$ bound to 70S *E. coli* ribosomes reacts covalently with proteins of the 50S subunit. The major reactions are with proteins L2 and L27. In the presence of poly(U), 70S-bound bromoacetyl-phenylalanyl-tRNA$^{Phe}$ can participate in peptide-bond formation with phenylalanyl-tRNA$^{Phe}$ or puromycin. Most of the products of these reactions are also found covalently attached to L2 and L27. Chloramphenicol and sparsomycin markedly inhibit the peptide-bond formation. These results strongly suggest that bromoacetyl-phenylalanyl-tRNA$^{Phe}$ can function as a normal peptidyl-tRNA and that the 50S proteins, L2 and L27, are located in the peptidyl-tRNA binding site. The side reactions of bromoacetyl-phenylalanyl-tRNA$^{Phe}$ are with one or more 50S proteins from the set L14–17, L6 and/or L11, and L26. These occur to a much less extent than the reactions with L2 and L27. Any functional significance of the side reactions is unknown.**

Previously we described the preparation and some of the properties of the peptidyl-tRNA analogue, *N*-bromoacetyl-phenylalanyl-tRNA$^{Phe}$ (BrAcPhe-tRNA). This compound was designed as a potential affinity label for the P site of *Escherichia coli* ribosomes (1). When bound to 70S ribosomes with poly(U) as messenger, this analogue reacted covalently with the 50S subunit. Initial studies suggested that there were two sites to which the reagent became covalently attached. BrAcPhe-tRNA was found associated with a single band in a one-dimensional polyacrylamide gel separation of the 50S proteins. This band contains proteins 3VI, 2IX, and 2XI [numbering according to Traut *et al.* (2)]. BrAcPhe-tRNA was also found associated with 23S rRNA. This finding implied either covalent attachment to the RNA or to a protein tightly bound to the RNA.

We have now been able to define more clearly the sites of covalent attachment of BrAcPhe. Two-dimensional gel electrophoresis has been used to obtain fairly unambiguous identification of the covalently modified 50S proteins. Also, improved procedures lead to a 30-fold increase in the amount of [$^3$H]phenylalanine covalently attached.

## MATERIALS AND METHODS

*Ribosomes and Affinity Labels.* 70S ribosomes, as well as 50S and 30S subunits, were prepared from *E. coli* A19 as described (1). Radioactively labeled Phe-tRNA$^{Phe}$ was prepared in three ways. For some experiments [$^3$H]Phe (50 Ci/mmol) or unlabeled Phe and unfractionated *E. coli* B tRNA were incubated with a crude supernatant from *E. coli* to give Phe-tRNA$^{Phe}$. This preparation contained over 99% unaminoacylated bulk tRNA, including unaminoacylated tRNA$^{Phe}$. It is the same method of preparation as used in our earlier work (1). We have also prepared purified unlabeled Phe and [$^3$H]Phe-tRNA$^{Phe}$ from *E. coli* B tRNA as described by Gillam and Tenner (3). This Phe-tRNA$^{Phe}$ contained less than 20% unaminoacylated tRNA$^{Phe}$. Finally, we prepared Phe-tRNA$^{Phe}$ from 80% pure yeast tRNA$^{Phe}$ (Boehringer–Mannheim) and unlabeled Phe or [$^3$H]Phe (50 Ci/mmol) with a purified supernatant from rat liver (4). This preparation contained less than 60% unaminoacylated tRNA.

BrAcPhe-tRNA and AcPhe-tRNA were prepared as described (1). Standard reaction mixtures of BrAcPhe-tRNA and ribosomes contained 400 $A_{260}$ units of 70S *E. coli* ribosomes, 20 $A_{260}$ units of poly(U), and 0.6 $\mu$Ci of [$^3$H]BrAcPhe-tRNA, all in 2 ml of buffer A(1) [10 mM Tris·HCl (pH 7.4)–10 mM $MgCl_2$–30 mM $NH_4Cl$]. The mixture was incubated for 30 min at 37°.

Other reactions contained unlabeled BrAcPhe-tRNA in an amount equivalent to 0.6 $\mu$Ci of [$^3$H]BrAcPhe-tRNA and 0.6 $\mu$Ci of [$^3$H]Phe-tRNA in addition to all other components named above. Reactions with [$^3$H]puromycin included 40 $A_{260}$ units of 70S ribosomes, 2 $A_{260}$ units of poly(U), and the unlabeled equivalent of 0.06 $\mu$Ci of [$^3$H]BrAcPhe-tRNA in 0.2 ml of buffer A. This mixture was also incubated for 30 min at 37°. [$^3$H]Puromycin (1 Ci/mmol) was then added to a final concentration of 0.2 mM and incubation was continued for 30 min more at 37°.

*Proteins and Two-Dimensional Gel Electrophoresis.* 50S ribosomal proteins and nucleic acids were separated by the addition of LiCl and urea to a final concentration of 2 M LiCl and 4 M urea (5). The solutions stood at 4° for 12–72 hr. Proteins were dialyzed against 4 M urea, and any remaining tRNA was digested with pancreatic ribonuclease.

Two-dimensional polyacrylamide-gel electrophoresis was performed by the method of Kaltschmidt and Wittman (6). The gels were stained in a solution of 0.05% Coomassie Brilliant Blue in 10% trichloroacetic acid (7). Within 1 hr the gels were sufficiently stained to permit identification of all proteins. Each protein was cut out at that time and trichloroacetic acid was removed from these gel pieces by ether extraction. The gel pieces were then oxidized in a Packard Tritium

Abbreviations: BrAcPhe-tRNA, *N*-bromoacetyl-phenylalanyl-tRNA$^{Phe}$; buffer A, 10 mM Tris·HCl (pH 7.4)–10 mM $MgCl_2$–30 mM $NH_4Cl$.

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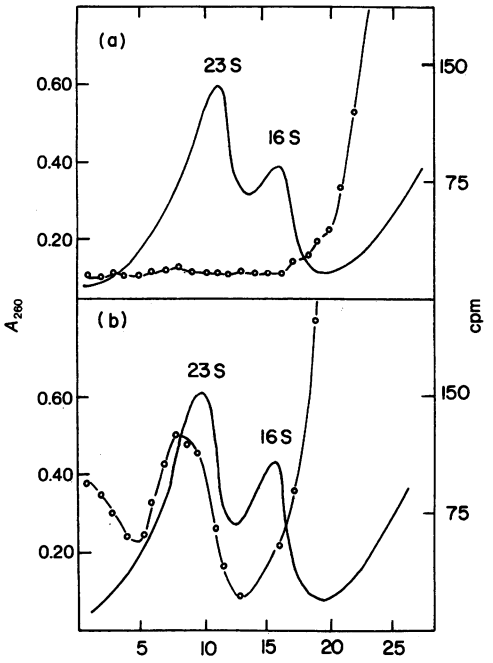


FIG. 1. Na dodecyl sulfate gradients of rRNA from ribosomes reacted with BrAcPhe-tRNA or AcPhe-tRNA. ——, $A_{260}$; O——O, cpm [$^3$H]Phe. 70S Ribosomes reacted with BrAcPhe-tRNA or AcPhe-tRNA were incubated in 1% Na dodecyl sulfate for 30 min at 37° and applied to 5–20% sucrose gradients in buffer A, which were made 0.1% in Na dodecyl sulfate. The gradient was centrifuged 4.5 hr at 45,000 rpm in an SW50 rotor. 250-μl fractions were collected. $A_{260}$ Was measured, and the fractions were analyzed for trichloroacetic acid-precipitable radioactivity as described in the legend to Table 1. (*a*) Ribosomes treated with AcPhe-tRNA; (*b*) ribosomes treated with BrAcPhe-tRNA.

Oxidizer and counted in a Packard Tri-Carb 3375. Alternatively, other two-dimensional gels were stained with amido black in 7% acetic acid (6).

*Antibiotics.* Chloramphenicol was obtained from Sigma Chemical Co. Sparsomycin was the generous gift of Drug Research and Development, Chemotherapy, NCI of the National Institutes of Health. [$^3$H]Puromycin was obtained from Schwarz–Mann.

TABLE 1. *2 M LiCl–4 M urea stripping of 70S ribosomal proteins*

| Hours (at 4°) | % of cpm with rRNA fraction |
|---|---|
| 12 | 41 |
| 24 | 26 |
| 48 | 16 |
| 72 | 7 |

70S Ribosomes reacted with BrAcPhe-tRNA in buffer A were brought to a final concentration of 2 M LiCl–4 M urea and incubated at 4° for the times indicated. The rRNA precipitate was removed by centrifugation at 10,000 × *g* for 10 min and dissolved. RNA remaining in the supernatant was digested with pancreatic RNase. The total protein and RNA fractions were precipitated with trichloroacetic acid, collected on 25-mm Millipore filters, and counted.

## RESULTS

*Nature of the 23S RNA-Associated BrAcPhe-tRNA.* As we reported earlier (1), after 50S particles from ribosomes reacted with BrAcPhe-tRNA were separated into a protein and an RNA fraction by treatment with LiCl–urea, trichloroacetic acid-precipitable radioactivity is found in both fractions. The radioactivity in the RNA fraction is in fact associated with the 23S RNA. This was shown by sucrose gradient sedimentation of ribosomes labeled with BrAcPhe-tRNA, which had subsequently been incubated in 1% Na dodecyl sulfate. Some radioactivity does cosediment with 23S RNA (Fig. 1). A control experiment in which the incubation mixture contained AcPhe-tRNA instead of BrAcPhe-tRNA showed no radioactivity associated with high-molecular-weight rRNA.

Since it is known that some 50S proteins bind very tightly to 23S RNA, we wanted to determine whether the RNA-associated BrAcPhe-tRNA was covalently linked to a protein bound to the RNA or to the RNA itself. The amount of radioactivity in the RNA fraction was determined as a function of the time of treatment in LiCl–urea. The results in Table 1 show that as the length of LiCl–urea treatment increases, the percent of trichloroacetic acid-precipitable radioactivity found in the RNA fraction decreases. This result indicates that the RNA-associated BrAcPhe-tRNA is linked to protein that can be removed from the RNA almost completely with prolonged LiCl–urea treatment. In our previous studies we had routinely continued the LiCl–urea treatment for 12–16 hr only, which does not completely remove this protein from the RNA. We also found that a high (greater than 5 mg/ml) concentration of ribosomes in LiCl–urea is necessary for complete separation of protein from rRNA.

*Identification of 50S Proteins Reacted with BrAcPhe-tRNA by Two-Dimensional Gels.* It was important to know whether the BrAcPhe-labeled protein(s) that was tightly bound to the 23S RNA is the same as the labeled protein previously found in the 50S protein fraction after 12–16 hr of LiCl–urea treatment of the 50S particles. The protein fraction obtained by a 48-hr LiCl–urea treatment of 50S subunits from ribosomes reacted with BrAcPhe-tRNA was analyzed by gel electrophoresis. Table 2 shows typical data from the analysis of a two-dimensional gel separation of 50S proteins from ribosomes reacted with BrAcPhe-tRNA (from crude *E. coli* tRNA). The majority of the radioactivity is found in two proteins: L2 and L27. L27 corresponds to the labeled protein we previously identified by a one-dimensional gel as 3VI, 2 IX, or 2 XI in the numbering system of Traut *et al.* (2).

Fig. 2 shows a photograph of a two-dimensional gel of labeled 50S proteins after 48-hr LiCl–urea treatment. The gel was stained with amido black. If the protein fraction from 50S ribosomes that have been treated with LiCl–urea for only 12 hr is analyzed on a two-dimensional gel, a similar pattern is obtained but the spot corresponding to L2 is missing. L2 must therefore be the labeled protein that was tightly bound to the 23S RNA.

As can be seen in Table 2, a few of the other 50S proteins also seem to be radioactively labeled with BrAcPhe-tRNA though to a less extent than L2 and L27. Due to the overlap of some of the protein spots on our gels there is some ambiguity about which proteins are actually labeled. In most of our experiments some radioactivity is found in some or all of the spots corresponding to proteins L14, L15, L16, and L17,

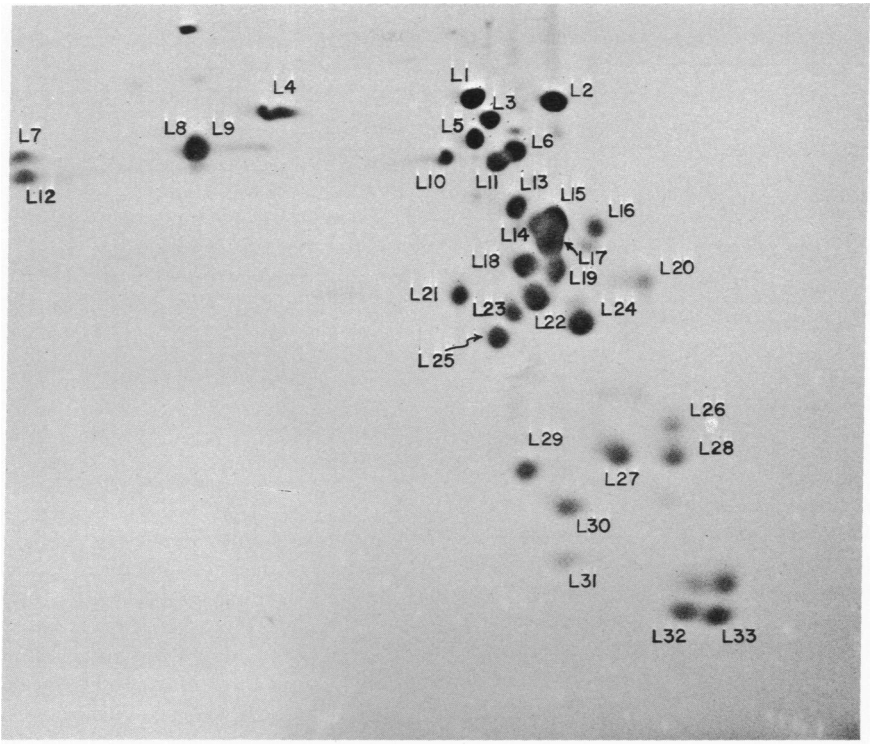


FIG. 2. Photograph of a two-dimensional gel separation of the 50S proteins performed according to Kaltschmidt and Wittman (6), and stained with amido black (7).

though possibly only one of these proteins is actually labeled by BrAcPhe-tRNA. The same is true of spots L6 and L11 (which overlap). Some radioactivity is also consistently found in spots L20 and L26.

Labeling experiments with BrAcPhe-tRNA prepared from purified yeast Phe-tRNA and purified *E. coli* Phe-tRNA also showed selective incorporation of radioactivity into proteins L2 and L27. Some variability was found in the relative amounts of radioactivity found in the two-dimensional protein spots corresponding to L2 and L27. This variability did not seem to depend on which preparation of tRNA was used to prepare the BrAcPhe-tRNA. It was probably at least partly due to incomplete removal of L2 from 23S rRNA in some experiments. When we worked with small samples, it was difficult to insure a ribosome concentration high enough for total stripping of proteins from rRNA. Minor differences between ribosome preparations may also have been a factor.

Table 3 shows the relative extent of labeling of those 50S proteins that react with BrAcPhe-tRNA expressed as a percentage of the total radioactivity above background in the two-dimensional gels. The results are averages from several two-dimensional electrophorograms of each BrAcPhe-tRNA preparation.

*BrAcPhe-tRNA Can Participate in Peptide Transfer.* A critical question is whether BrAcPhe-tRNA can bind in a functionally correct way and undergo peptide transfer. Experiments were done in which unlabeled BrAcPhe-tRNA and [$^3$H]Phe-tRNA were added simultaneously to ribosomes in the standard incubation mixture. In these experiments radioactivity becomes covalently linked to the ribosome (1). For covalent attachment of radioactivity to the ribosome to occur in this case, peptidyl transfer must occur between these two tRNA moieties. In a typical experiment, over 4000 cpm of [$^3$H]Phe was recovered from 50S protein spots on a two-dimensional gel. The relative extent of labeling of the 50S proteins under these conditions is shown in Table 3. The results are similar to those obtained with [$^3$H]BrAcPhe-tRNA, though the relative labeling of L14–L17 seems slightly greater and small amounts of radioactivity are found in proteins L5

TABLE 2. *Data from a typical two-dimensional gel separation of 50S proteins from ribosomes reacted with [$^3$H]BrAcPhe-tRNA*

| Protein | cpm | Protein | cpm |
|---|---|---|---|
| L1 | 110 | L18 | 155 |
| L2 | 3010 | L19 | 150 |
| L3 | 300 | L20 | 305 |
| L4 | 254 | L21 | 78 |
| L5 | 105 | L22 | 140 |
| L6 | 370 | L23 | 150 |
| L7 + L12 | 85 | L24 | 220 |
| L8 + L9 | 90 | L25 | 180 |
| L10 | 195 | L26 | 585 |
| L11 | 325 | L27 | 2175 |
| L13 | 89 | L28 | 138 |
| L14 | 96 | L29 | 117 |
| L15 | 95 | L30 | 257 |
| L16 | 510 | L31 | 151 |
| L17 | 205 | L32 | 98 |
| | | L33 | 105 |

Two-dimensional gels were stained with Coomassie Blue and the protein spots were analyzed for radioactivity. Background was not subtracted. *Underlined incorporations* are those deemed significantly above background.

TABLE 3. *Pattern of BrAcPhe reaction with 70S ribosomes*

| Protein | [$^3$H]BrAcPhe-tRNA (%) | [$^1$H]BrAcPhe-tRNA + [$^3$H]-Phe-tRNA (%) | [$^1$H]BrAcPhe-tRNA + [$^3$H]-puromycin (%) |
|---|---|---|---|
| L2 | 54 ± 15 | 41 ± 15 | 39 |
| L5 | — | 4 ± 4 | 9 |
| L10 | — | 4 ± 4 | — |
| L6 + L11 | 2 ± 1 | 4 ± 10 | 12 |
| L14–L17 | 8 ± 4 | 22 ± 8 | 22 |
| L20 | 2 ± 1 | 1 ± 1 | — |
| L26 | 6 ± 5 | 2 ± 3 | — |
| L27 | 29 ± 22 | 22 ± 23 | 19 |

Two-dimensional gels of total 50S proteins obtained from ribosomes reacted with BrAcPhe-tRNA were analyzed for radioactivity. The cpm above background present in the proteins listed were expressed as a percent of the cpm above background in the whole gel. The results are averaged over several experiments and the standard deviations are indicated, except for the experiment with [$^3$H]puromycin. The results with puromycin are based on total amounts of incorporation, which are only a few percent of those shown in Table 2. This was necessitated by the lower specific activity of puromycin available and its cost.

and L10. The predominant labeled proteins are L2 and L27. That covalent attachment of [$^3$H]Phe does in fact occur through peptide-bond formation between unlabeled BrAcPhe-tRNA and [$^3$H]Phe-tRNA is confirmed by the fact that incorporation of radioactivity into ribosomal protein is inhibited by chloramphenicol and sparsomycin (Table 4). These two antibiotics are generally believed to inhibit peptide-bond formation. When nonradioactive BrAcPhe-tRNA was allowed to react with ribosomes in the presence of radioactive puromycin, once again radioactive labeling of 50S ribosomal proteins was observed (see Table 3.) Due to the low specific activity of puromycin used, the total radioactivity incorporated into protein was very low. However, the pattern of labeling observed was similar to that obtained with [$^3$H]BrAcPhe-tRNA. Again, the covalent attachment of radioactivity to ribosomes would require correct placement of BrAcPhe-tRNA in the P site so that peptide-bond formation could occur with puromycin.

## DISCUSSION

We have shown by two-dimensional gel analysis that the 50S ribosomal proteins L2, L27, and possibly one or more of the proteins L14–L17 correspond to the site(s) of covalent attachment of [$^3$H]BrAcPhe-tRNA to the ribosome. To a much less extent, L6 and/or L11, L20, and L26 are also labeled by this reagent. L2 and L27 are the major labeled components containing over 75% of the radioactivity found associated with proteins on our gels.

L2 and L27 also contain most of the $^3$H label when nonradioactive BrAcPhe-tRNA and radioactive Phe-tRNA were incubated simultaneously with the ribosome. Incorporation of radioactivity into ribosomal proteins occurs by way of peptide-bond formation between these two tRNA species, as shown by

TABLE 4. *Use of antibiotics to inhibit covalent labeling by nonradioactive BrAcPhe-tRNA and [$^3$H]Phe-tRNA*

| Additions | cpm in total protein fraction | % Inhibition |
|---|---|---|
| None | 3440 | — |
| 1.0 mM Chloramphenicol | 501 | 85 |
| 0.1 mM Sparsomycin | 249 | 93 |

70S Ribosomes were labeled with nonradioactive BrAcPhe-tRNA and [$^3$H]Phe-tRNA. After incubation at 37°, the ribosomes were treated with 2 M LiCl–4 M urea to strip the proteins. rRNA precipitates were removed by centrifugation and protein supernatants were treated with ribonuclease. Trichloroacetic acid-precipitable radioactivity in the protein fraction was then determined on Millipore filters.

inhibition of this radioactive labeling in the presence of the peptidyl transferase inhibitors, chloramphenicol and sparcomycin. There is no way to tell from this experiment alone whether BrAcPhe-tRNA alkylation of L2 and L27 precedes or follows peptide-bond formation. However, the fact that L2 and L27 are the main products in both types of reactions is striking. These two proteins are also the main products in the labeling reaction containing nonradioactive BrAcPhe-tRNA and [$^3$H]puromycin. Therefore, we propose that L2 and L27 are located in the P site of the 50S ribosome.

The significance of the minor labeled protein components L6 and/or L11, L14–L17, L20, and L26 must also be considered. There are several possibilities for the occurrence of these side reactions. They may result from nonspecifically bound BrAcPhe-tRNA. The 3′ end of a peptidyl-tRNA may have considerable flexibility or the ribosomes themselves may be structurally or functionally heterogeneous. There may exist different functionally significant modes of P-site binding, for example, precursors or successors to the complex in which the peptide bond is formed. Finally, the minor reaction products may result from initially unreacted BrAcPhe-tRNA, which reacts at some subsequent point in handling of the sample.

In light of the fairly specific reaction between BrAcPhe-tRNA and proteins L2 and L27 it is interesting to survey what else is known about these proteins. The proteins of the 50S particle are in general less reactive towards added enzymes or organic reagents than those of the 30S particle (8–10; 12; Litman, D. & Cantor, C. R., in preparation). One of the most reactive 50S proteins toward fluorescene-isothiocyanate (9) and trypsin digestion (8) is L2. Both L2 and L27 react with *N*-ethylmaleimide (10) and both are enzymatically iodinated (Litman & Cantor, in preparation).

Traugh and Traut (11) identified L2 as the major 50S protein phosphorylated by rabbit-muscle kinase. L2 is the most rapidly phosphorylated species in the 70S ribosome. They suggest that destruction of 70S ribosome protein-synthesizing activity by rabbit-muscle kinase is probably due to phosphorylation of protein L2. Protein L3 from the 50S subunit of *B. stearothermophilus* ribosomes corresponds directly to protein L2 from *E. coli* (12–14). Fahnestock *et al.* (15) have shown that although L3 from *B. stearothermophilus* is not required for *in vitro* binding of other proteins in reconstitution of the 50S particle, it is necessary for assembly of active 50S subunits.

There is much less known about the functional significance of L27. It is present in less than one copy per ribosome, while L2 is a unit protein (16). The above results plus the ease of reaction of L2 with BrAcPhe-tRNA encourage speculation that this protein may be intimately linked with the peptidyl transferase activity of the ribosome. However, this fact remains unproven.

BrAc-aminoacyl-tRNAs will be useful in further studies. Several approaches immediately suggest themselves. It is important to determine whether such reactive tRNAs will label ribosomes in a "physiological" protein-synthesizing system *in vitro* with natural mRNA. Also BrAc-aminoacyl-tRNA should be helpful in studying possible distortion of the P site by antibiotics or solvent. A final problem is the identification of the BrAcPhe-tRNA reactive moiety or moieties in proteins L2 and L27.

After this work was completed, the use of the *N*-bromoacetyl analogue of chloramphenicol to label the 50S proteins in the chloramphenicol binding site was reported (17). This reagent also reacts primarily with proteins L2 and L27.

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