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Cell-free Synthesis of a Specific Lipoprotein of the *Escherichia coli* Outer Membrane Directed by Purified Messenger RNA

(immunoassay/BrCN cleavage/protein identification)

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ABSTRACT **A specific lipoprotein of the *E. coli* outer membrane has been synthesized in a cell-free system directed by purified messenger RNA. The mRNA for the lipoprotein was purified as 7S RNA about 250-fold from exponentially growing cells. Protein synthesis of the cell-free system was totally dependent upon the addition of the purified mRNA. The product of the cell-free system was identified as the specific lipoprotein by immunoprecipitation and by peptide mapping.**

The envelope of *Escherichia coli* has a peculiar lipoprotein consisting of 58 amino-acid residues. The entire chemical structure of the lipoprotein has been established, and an unusual amino acid, glycerycysteine [*S*-(propane-2′,3′-diol)-3-thio-2-aminopropionic acid] has been found at the amino-terminal end, to which two fatty acids are attached by two ester linkages and one fatty acid by an amide linkage (1–3). The lipoprotein exists in two different forms in the *E. coli* envelope (4, 5), a free form, and a bound form which is covalently linked to the peptidoglycan (6, 7). There is twice as much of the free form as the bound form (4), and both forms are located exclusively in the outer membrane (8, 9). The number of the lipoprotein molecules per cell has been estimated to be 5×10^5 and 2.5×10^5 for the free and the bound forms, respectively (4, 10). Thus, it appears to be the most abundant protein in *E. coli*. Recently, we have proposed a molecular assembly model of the lipoprotein in the outer membrane, which provides a passive diffusion pore through the outer membrane (11).

The biosynthesis of the lipoprotein has also been investigated (4, 9, 12, 13); the free form is synthesized first, and the newly synthesized free form is very efficiently incorporated into the outer membrane without being accumulated in the inner membrane (4, 9). A part of the free form is then converted to the bound form. A dynamic equilibrium between the two forms has been suggested (4). Furthermore, striking features of the *in vivo* biosynthesis of this membrane protein are (*a*) the extraordinary stability of the mRNA for the lipoprotein, i.e., a half life of 11.5 min (12, 13), (*b*) the unusual puromycin resistance of the biosynthesis of the lipoprotein (13), and (*c*) the exclusive biosynthesis of the lipoprotein in cells starved of histidine (12).

In the present communication, mRNA for the lipoprotein has been highly purified by taking advantages of the following characteristics: (*a*) the mRNA is highly stable, (*b*) the size of the mRNA is assumed to be much smaller than that of most *E. coli* mRNAs if the mRNA is monocistronic, and (*c*) the amount of the mRNA is also assumed to be abundant. The mRNA thus purified has been found to be biologically active, and the product in a cell-free system directed by the mRNA has been identified to be the lipoprotein by immunological assay and by peptide mapping.

Abbreviation: TCA, trichloroacetic acid.

MATERIALS AND METHODS

Cells. E. coli CP78 (*thi*$^-$, *arg*$^-$, *his*$^-$, *thr*$^-$, *leu*$^-$, RC$^+$) was grown in tryptone broth containing 10 g/liter of bactotryptone (Difco), 10 g/liter of yeast extract (Difco), and 5 g/liter of NaCl, at 37° with use of Hi-density Fermentor (Lab-line). At the middle of the log phase, chloramphenicol was added to the culture to a final concentration of 50 μg/ml. The culture was then immediately chilled with ice. About 48 g of the cells were collected from three separate cultures of 4 liters each.

Cell-Free System of Protein Synthesis. Cell-free protein synthesis was carried out in 50 mM Tris·HCl (pH 7.8), 8.3 mM $Mg(CH_3COO)_2$, 85 mM NH_4Cl, 1 mM dithiothreitol, 2.1 mM phosphoenolpyruvate, 0.43 mM ATP, 0.043 mM GTP, 0.043 mM amino acids (histidine, tryptophan, proline, glycine, methionine, and phenylalanine were omitted from the usual 20 amino acids), 0.053 mM palmitic acid (see *Discussion*), 85 μg/ml of pyruvate kinase, 20 μCi/ml of [^{35}S]methionine (111 Ci/mmol; New England Nuclear Corp.), 68 A_{260} units/ml of preincubated S-30 extract, and various amounts of messenger RNA fraction. In the peptide mapping experiment, [^{14}C]arginine (312 mCi/mmol; Schwarz/Mann) was used instead of [^{35}S]methionine, and arginine in the mixture of 14 amino acids was replaced by methionine. The reaction was carried out at 33°. In order to examine amino-acid incorporation into the protein fraction, 10-μl aliquots of the reaction mixture were withdrawn at intervals, put onto a Whatman 3MM filter disc, and the disc was put into 5% trichloroacetic acid (TCA) solution. The TCA solution was then incubated in a boiling-water bath for 30 min. The disc was rinsed with 5% TCA and dried with acetone. The radioactivity left on the disc as hot TCA-insoluble material was determined in a liquid scintillation counter.

The preincubated S-30 extract was prepared from *E. coli* Q13 as described previously (14).

Polyacrylamide Gel Electrophoresis. The purity of RNA preparations was examined by slab gel electrophoresis with 7.5% acrylamide gel according to the method of Studier (15), except that sodium dodecyl sulfate was omitted. The gel was stained with methylene blue (16).

The protein synthesized in the cell-free system was analyzed by disc gel electrophoresis, as described previously (17, 18).

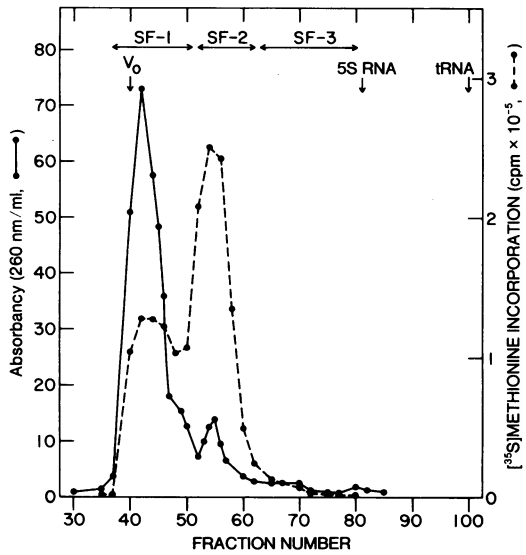

FIG. 1. Purification of the mRNA for the lipoprotein by gel filtration on Sephadex G-200. RNA was extracted by phenol from 48 g of exponentially growing cells. The mRNA for the lipoprotein was purified by NaCl fractionation, gel filtration on Sephadex G-100, and 5–20% sucrose density gradient centrifugation. The mRNA fraction from the sucrose density gradient centrifugation was further purified by gel filtration on Sephadex G-200; 2 ml of the RNA fraction in 0.1 M KCl were applied to a Sephadex G-200 (superfine) column (1.2 × 50 cm) equilibrated with 0.1 M KCl, and fractions of 0.7 ml were collected at the flow rate of 3.2 ml/hr. For the assay of the mRNA activity, 20 μl of each fraction were used for the cell-free system (50 μl) described in *Materials and Methods*. After 15-min incubation at 33°, radioactivity incorporated into hot TCA-insoluble material was measured. V_0, 5S RNA, and tRNA indicate the positions of the void volume of the column, 5S RNA, and tRNAs from *E. coli* B, respectively. SF-1, SF-2, and SF-3 are three pooled RNA fractions.

Immunoprecipitation. The product of the cell-free system was also identified by an immunoprecipitation method specific for the lipoprotein, which has been developed in our laboratory (Inouye, Takeishi, Hirashima, and Inouye, manuscript in preparation) as follows: Four-hundredths milliliter of the reaction mixture of the cell-free system was mixed with 0.36 ml of the purified lipoprotein (0.56 mg/ml) in 0.14% sodium dodecyl sulfate and 0.1 M sodium phosphate buffer (pH 7.0). Then 0.1 ml of anti-lipoprotein serum was added to the mixture. The final mixture was incubated overnight at 4°. The precipitate thus formed was collected by centrifugation and washed three times with 1.5 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 0.9% NaCl, 0.1 mM EDTA, and 0.1% sodium dodecyl sulfate.

Peptide Mapping. The product, labeled with 5 μCi of [^{14}C]-arginine for 45 min at 33° in the 1-ml reaction mixture of the cell-free system, was subjected to immunoprecipitation, as described above. The precipitate was solubilized in 1% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer (pH 7.0). The solution was then subjected to disc gel electrophoresis. After electrophoresis, the gel portions corresponding to the location expected for the lipoprotein using bromphenol blue as a marker, were cut out. The radioactive product was extracted from the gels with water. To the extract, 2 mg of the purified lipoprotein and sodium dodecyl sulfate (final concentration of 0.1%) were added, and the radioactive product was

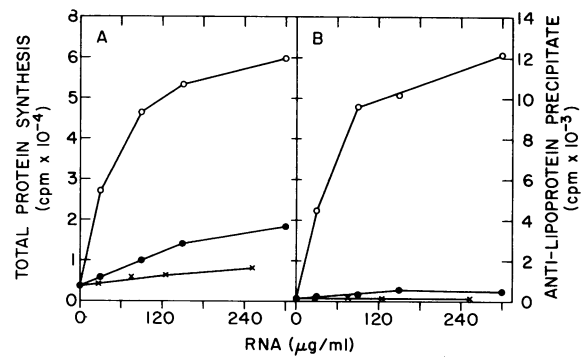

FIG. 2. Messenger RNA activity of the RNA fractions from Sephadex G-200 gel filtration. (A) Incorporation of [^{35}S]methionine into hot TCA-insoluble material in the cell-free system directed by various amounts of the RNA fractions. The reaction mixtures of 100 μl (see the composition in *Materials and Methods*) were incubated at 33° for 30 min. The hot TCA-insoluble radioactivity was measured in 10-μl portions of the reaction mixture. (B) Immunoprecipitation of the *in vitro* products by anti-lipoprotein serum. Immunoprecipitation was carried out with 40 μl of the reaction mixture as described in *Materials and Methods*. Control experiments with serum from nonimmunized rabbits were also carried out; the values from the control experiments were subtracted from those of the experimental immunoprecipitates. All values in the figure are calculated for 10 μl of the reaction mixture. ●, SF-1; O, SF-2; ×, SF-3 RNA.

precipitated by adding 2 volumes of acetone. The pellet was washed three times with 60% acetone. Cyanogen bromide cleavage was carried out overnight at room temperature with 6 mg of BrCN in 80% formic acid. The product was then lyophilized and suspended in 0.5 ml of water. The insoluble material corresponding to the amino-terminal half of the lipoprotein was removed by centrifugation. About 90% of the total radioactivity was recovered in the supernatant and a part of the supernatant was applied to a thin cellulose MN300 plate (CEL 300, Macherey Nagel Co.) for peptide mapping. Separation of peptides was carried out first by chromatography with a solvent of pyridine:acetic acid:water:*n*-butanol (60:18:72:90) followed by high voltage electrophoresis at pH 3.5.

RESULTS

Purification of mRNA for the Lipoprotein. Forty-eight grams of exponentially growing cells were lysed with egg-white lysozyme and sodium dodecyl sulfate. The mRNA for the lipoprotein was purified from the lysate by (*a*) phenol extraction, (*b*) NaCl fractionation, (*c*) gel filtration on Sephadex G-100, (*d*) 5–20% sucrose density gradient centrifugation, and (*e*) gel filtration on Sephadex G-200. The detailed procedures will be published elsewhere (Wang, Hirashima, and Inouye, manuscript in preparation). Fig. 1 shows the results of the last gel filtration on Sephadex G-200. About 85% of the total RNA applied appeared at or immediately following the void volume, while about 9.5% of the total RNA was recovered as a small peak in fraction number 55. However, when the ability of the RNA fractions to incorporate amino acids into hot TCA-insoluble material in the cell-free system was examined, it was found that the RNA from the smaller peak has much higher mRNA activity than the RNA from or near the void volume as shown in Fig. 1.

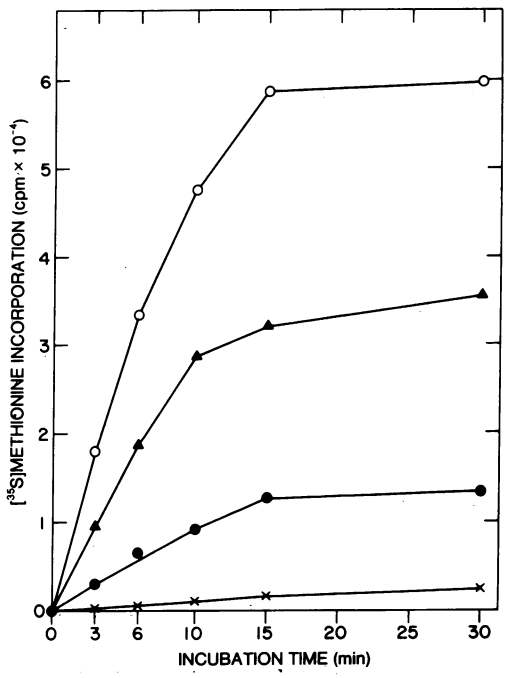

FIG. 3. Time course of *in vitro* protein synthesis directed by various amounts of SF-2 RNA from gel filtration on Sephadex G-200. The reaction mixture is the same as described in Fig. 2. At the intervals indicated in the figure, 10-μl aliquots of the reaction mixture were withdrawn to measure radioactivity incorporated into hot TCA-insoluble material. ○, 150 μg/ml; ▲, 60 μg/ml; ●, 15 μg/ml of SF-2 RNA and; ×, no RNA was added to the reaction mixtures.

Identification of the mRNA Specific for the Lipoprotein. In order to determine the position of the mRNA for the lipoprotein in Fig. 1, we pooled RNA fractions into three portions, SF-1, SF-2, and SF-3 as shown in Fig. 1, and the *in vitro* products directed by these fractions were examined immunologically with the use of anti-lipoprotein serum. Fig. 2A shows [^{35}S]methionine incorporation into hot TCA-insoluble material in the cell-free system directed by different amounts of the pooled RNA fractions. SF-2 RNA was found to have about 10 and 20 times higher specific activity as measured by protein synthesis than SF-1 or SF-3 RNA, respectively (Fig. 2A). When the products were analyzed by immunoprecipitation, it was clear that SF-2 RNA is directing the formation of the lipoprotein *in vitro* (Fig. 2B). The reactions shown in Fig. 2 reached almost maximal levels at 90 μg/ml of SF-2 RNA. At this step, we have achieved about 250-fold purification over the original crude phenol extract.

The recovery of radioactivity in the immunoprecipitate shown in Fig. 2B is about 20% of the radioactivity incorporated into hot TCA-insoluble material (compare Fig. 2B with Fig. 2A). In this experiment the immunoprecipitate was washed with 0.1% sodium dodecyl sulfate. However, if the precipitate was washed with 0.01% sodium dodecyl sulfate, the recovery in the immunoprecipitate is found to be as high as the recovery in hot TCA-insoluble material. Since the present immunoprecipitation method has been proved to be highly specific to the lipoprotein (Inouye, Takeishi, Hirashima, and Inouye, manuscript in preparation), these results indicate that the product with SF-2 RNA consists mainly of the lipoprotein.

Fig. 3 shows time course experiments with different amounts of SF-2 RNA. Incorporation of [^{35}S]methionine into hot TCA-insoluble material continues for at least 15 min, and the rates of incorporation increase linearly with the amounts of SF-2 RNA added to the cell-free system. This result indicates that the present cell-free system is entirely dependent upon the mRNA added.

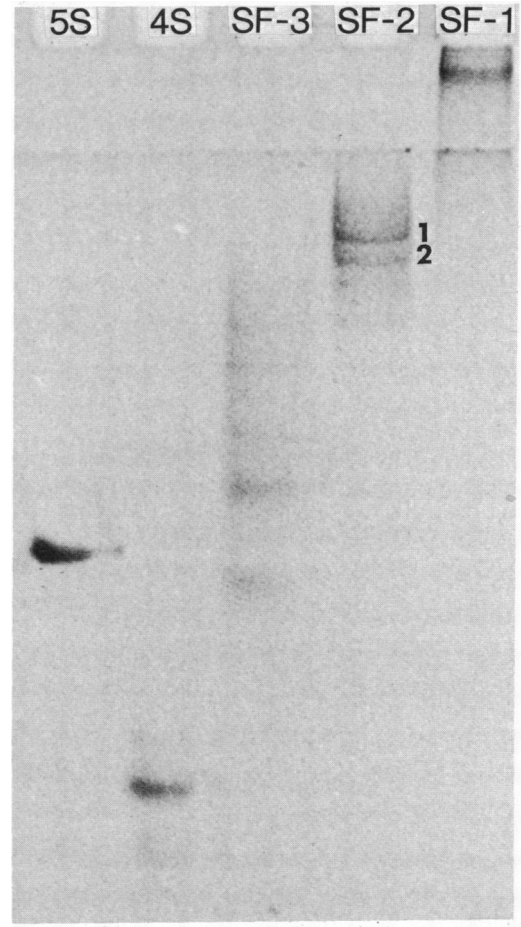

FIG. 4. Slab gel electrophoresis of RNA fractions from gel filtration on Sephadex G-200. About 20 μg of each fraction were applied for electrophoresis. Purified wheat-germ 5S RNA (10 μg) and 4S tRNA^{Phe} (10 μg) were also applied as standards. Electrophoresis was carried out on a 7.5% gel (15). Mobility of RNA is from the top to the bottom.

The molecular weight of SF-2 RNA was estimated from Fig. 1 to be about 70,000, which corresponds to 225 nucleotides. When SF-2 RNA was analyzed by acrylamide slab gel electrophoresis followed by staining with methylene blue, it was separated into two closely adjacent bands, 1 and 2, at about a 7S position as shown in Fig. 4. SF-1 and SF-3 RNA did not contain these RNA bands. In SF-1 RNA, a main component was found in the spacer gel as a single band, which is assumed to be 16S RNA, whereas the SF-3 RNA fraction was separated into a number of bands, all of which were found to be smaller than those in SF-2 RNA (Fig. 4). The sizes of band 1 and 2 RNAs were estimated from their relative mobilities to purified wheat germ 5S RNA (120 nucleotides) (19) and 4S tRNA^{Phe} (76 nucleotides) (20) to be approximately 250 and 230 nucleotides, respectively. These values are in good agreement with that obtained from gel filtration on Sephadex G-200 (Fig. 1). Judging from the density of the staining, the amount of band 1 RNA appears to be slightly greater than that of band 2 RNA. It is not yet known whether

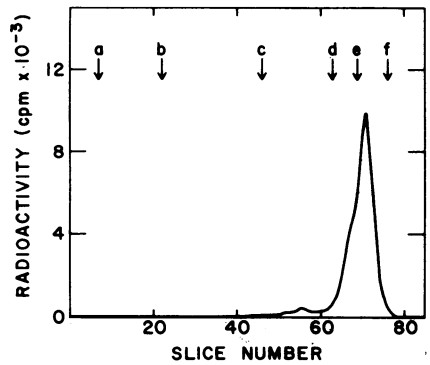

FIG. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the product in the cell-free system directed by SF-2 RNA. The reaction mixture (100 μl) was the same as described in Fig. 2, except that 90 μg/ml of SF-2 RNA were used. After 45-min incubation at 33°, the reaction mixture was treated with alkali and then the TCA-insoluble material was subjected to gel electrophoresis with the use of 10% gel (17, 18). Arrows with letters indicate positions of the internal molecular weight standards (18); (a) dimer; (b) monomer of Dns-bovine serum albumin; (c) dimer; (d) monomer of hen-egg white lysozyme; (e) cytochrome *c*; (f) Dns-insulin.

one or both of these RNAs have mRNA activity for the lipoprotein.

Further Identification of the In Vitro Product. The *in vitro* product was identified by immunoprecipitation, as described above, to be the lipoprotein. In Fig. 5, the *in vitro* reaction mixture directed by SF-2 RNA was treated with alkali to degrade aminoacyl-tRNA, followed by TCA precipitation, and then analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. More than 95% of total radioactivity migrated as a single peak between the internal molecular weight standards, cytochrome *c* (*e*) and Dns-insulin (*f*). The apparent molecular weight of the *in vitro* product is slightly larger than that of the *in vivo* product. The reason for this difference is unknown at present; the *in vitro* product may indeed be a longer peptide than the *in vivo* product, or alternatively, an incomplete modification of the amino-terminal end of the *in vitro* product may cause an abnormal mobility in the gel. It is noteworthy that a small shoulder is to be observed at the higher molecular weight side of the peak. This is possibly due to a dimer of the lipoprotein, which is formed by an S–S bridge between the amino-terminal cysteine residues of two unmodified lipoproteins produced in the cell-free system.

A more definite proof that the *in vitro* product is the lipoprotein is provided by peptide mapping. Since the lipoprotein is extremely resistant to proteases, the *in vitro* product labeled with [^{14}C]arginine was cleaved by cyanogen bromide. The *in vitro* product was mixed with nonradioactive purified lipoprotein as carrier and purified by immunoprecipitation, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 6, radioactive spots derived from the *in vitro* product (Fig. 6A) are in very good agreement with ninhydrin-stained spots derived from the *in vivo* product (Fig. B6). We have identified these spots as follows: (Inouye, Takeishi, Hirashima, and Inouye, manuscript in preparation); spot 1, Ala-Thr-Lys-Tyr-$\underset{53}{\text{Arg}}$-Lys; spot 2, $\underset{32}{\text{Arg}}$-Ser-Asp-Val-Gln-Ala-Ala-Lys-Asp-Asp-Ala-Ala-$\underset{58}{\text{Arg}}$-Ala-Asn-Glu-$\underset{52}{\text{Arg}}$-Leu-Asp-Asn-Hse; and spot 3, uncleaved peptide from residue 32 to 58 (spot 1 peptide + spot 2 peptide). Spot 4 has

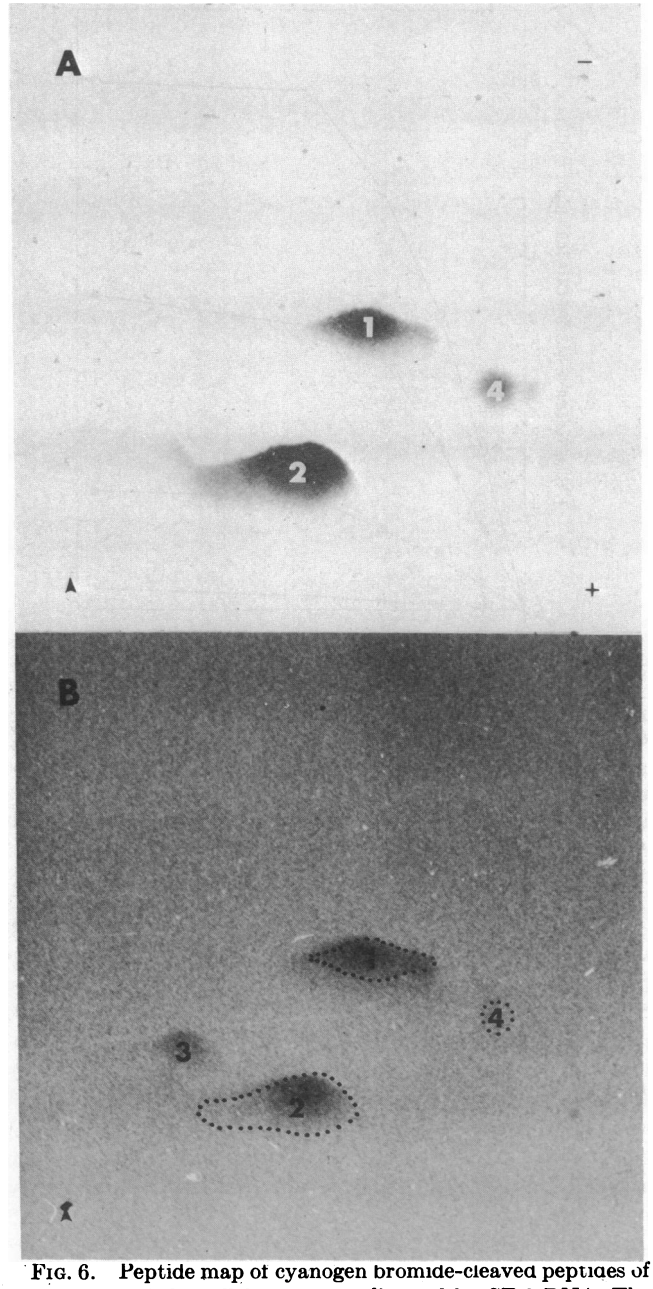

FIG. 6. Peptide map of cyanogen bromide-cleaved peptides of the product of the cell-free system directed by SF-2 RNA. The *in vitro* product was labeled with [^{14}C]arginine and purified as described in *Materials and Methods*. (A) autoradiogram, and (B) ninhydrin-stained map. The autoradiogram (A) specified by the dotted lines is superimposed on the ninhydrin-stained map (B). Small arrows indicate the positions of the origin. The peptides were separated first by chromatography and then by electrophoresis.

not been completely identified, but judging from data so far obtained, it appears to be the same peptide as spot 1 except that the tyrosine residue is modified (possibly brominated during cyanogen bromide cleavage). Since the *in vitro* product was labeled with [^{14}C]arginine, and spot 1 and 4 peptides each contain only one arginine residue as underlined above, in contrast to three residues in spot 2 peptide, the total radioactivity in spot 1 + 4 is expected to be a third of the radioactivity of spot 2. Radioactivities of these spots were found to be 307, 1038, and 101 cpm for spots 1, 2, and 4, respectively. Thus, the ratio of spot 1 + 4 to spot 2 is calculated to be

1/2.5, which is also in good agreement with the theoretical value, 1/3.

The *in vitro* product did not give rise to spot 3 (Fig. 6A). This is possibly due to decreased susceptibility of the purified *in vivo* product to cyanogen bromide cleavage. Such resistance may be caused by partial oxidation of methionine residues during the purification of the lipoprotein from *E. coli* cells.

DISCUSSION

Cell-free biosynthesis of a specific *E. coli* membrane protein directed by purified mRNA has been demonstrated. The mRNA for the lipoprotein of the *E. coli* outer membrane has been purified about 250-fold as 7S RNA from exponentially growing cells. When analyzed by slab gel electrophoresis, the RNA fraction was found to be separated into two closely adjacent bands (Fig. 4); one consists of about 250 (band 1) and the other consists of about 230 nucleotides (band 2). At present it is unknown whether one or both of these RNAs have the mRNA activity for the lipoprotein. It is possible that one is processed from the other. Although a very minor RNA band is observed in the SF-2 RNA fraction (Fig. 4), it is unlikely that stimulation of the lipoprotein synthesis by SF-2 RNA is due to the minor component, because the SF-3 RNA fraction contains exactly the same band as the minor RNA of SF-2 RNA, and SF-3 RNA does not stimulate the lipoprotein synthesis (Fig. 2B).

Since the lipoprotein consists of 58 amino-acid residues (1), at least 180 nucleotides are required for its biosynthesis [$= 3 \times (58 + 1$ for initiation codon and $+1$ for termination codon)]. Thus, it appears that the mRNA for the lipoprotein has 50 to 70 nontranslated nucleotides. These extra nucleotides are possibly used for the ribosome recognition site at the 5′ end of the mRNA and for the termination signal at the 3′ end. It has been reported that the mRNA for β-galactosidase of *E. coli* has 38 extra nucleotides before the AUG initiator codon (21). In the case of the mRNA for anthranilate synthetase of *E. coli trpE* gene product, the initiator codon has been shown to be preceded by a "leader" sequence of about 150 nucleotides (22). Therefore, it will be extremely interesting to determine the nucleotide sequence of the present mRNA for the lipoprotein. The structural study of the mRNA may reveal specific feature(s) of an mRNA for a membrane protein and may elucidate the reason for the unusual stability of the mRNA. These problems are now under investigation.

From the facts that there are 7.5×10^5 lipoprotein molecules in a cell and that the lipoprotein consists of 58 amino acids (10), the number of the mRNA molecules for the lipoprotein in a cell can be estimated as follows: It is known that the rate of translation of mRNA at 37° is about 60 nucleotides (= 20 amino acids)/sec (23–25). Thus, one mRNA molecule for the lipoprotein can produce 1800 lipoprotein molecules in one generation time of 45 min at 37°, assuming that one mRNA molecule is always translated by two ribosomes. Therefore, in order to produce 7.5×10^5 lipoprotein molecules in one generation time, about 400 mRNA molecules ($= 7.5 \times 10^5/1800$) are required in a cell.

The product of the cell-free system directed by SF-2 RNA has been clearly shown to be the lipoprotein by both immunoprecipitation and by peptide mapping. It should be noted that the *in vitro* product has the same carboxyl terminal peptide, $\overset{53}{\text{Ala-Thr-Lys-Tyr-Arg-Lys}}\overset{58}{}$, and has been isolated in a good recovery after cyanogen bromide cleavage of the *in vitro* product (spot 1 in Fig. 6A). At present it is not yet known whether the amino-terminal end is modified or not in the cell-free system. Preliminary experiments show that palmitic acid is not required for *in vitro* synthesis of the lipoprotein, and it is only slightly stimulatory. No incorporation of radioactive palmitic acid into the protein has been observed. Since it has been reported that in a cell-free protein synthesizing system, the initiator amino acid, *N*-formyl-methionine is still remaining at the N-terminus of *in vitro* products (26, 27), it is possible that *N*-formyl-methionine must be removed prior to the attachment of palmitic acid to the amino-terminal cysteine residue. At least three additional enzyme systems are thought to be required for the modification of the amino terminal end of the lipoprotein: (*a*) formation of an amide linkage between a fatty acid and the amino group of the amino-terminal cysteine residue, (*b*) formation of a thioether linkage between the amino terminal cysteine residue and a glycerol, and (*c*) formation of two ester linkages between the glyceryl group and two fatty acids. In addition to these modification reactions, it is of interest to investigate the interaction of the *in vitro* product with the membrane fraction and in the cell-free system.

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