chains on oxygen binding, it will be necessary to study the purified cross-linked species.

Within the β cleft of deoxyhemoglobin the distances between pairs of amino groups on opposite β chains are as follows (Arnone, 1972; Benesch et al., 1975): Lys-82···Lys-82, 8.1 Å; Lys-82···Val-1, 11 Å; Val-1···Val-1, 18 Å. In oxyhemoglobin, the first two spans are shorter. From molecular models we estimate the following distances between N atoms attached to opposite ends of fully extended dicarboxylic acids: $-(O{=})CCH{=}CHC(=O)-$, 6.8 Å; $-(O{=})C(CH_2)_2C(=O)-$, 6.8 Å; $-(O{=})C(CH_2)_4C(=O)-$, 9.2 Å. Comparison of these values with the distances between amino groups across the β cleft rules out the possibility of a four-carbon bridge between the valine N termini. Of the other two potential sites for cross-linking, a Lys-82 → Lys-82 bridge would be favored since its span is the shorter one.

With the disalicyl diesters, it has also been found (Zaugg, 1978) that longer bridges, for example, with adipate and suberate linkages, are *not* formed when oxyhemoglobin is treated with the respective diaspirins.[3] Evidently the $-NH_2$ sites most susceptible to cross-linking are spanned best by a four-carbon bridge. Clearly, the β portal of hemoglobin is sharply selective in the structures that have steric access to this cavity. This selectivity is an attractive feature in the potential use of diaspirins as antisickling agents.

References

Arnone, A. (1972) *Nature (London) 237*, 146–149.
Benesch, R., Benesch, R. E., Yung, S., & Edalji, R. (1975) *Biochem. Biophys. Res. Commun. 63*, 1123–1129.
Bookchin, R. M., & Nagel, R. L. (1971) *J. Mol. Biol. 60*, 263–270.
Bridges, K. R., Schmidt, G. T., Jensen, M., Cerami, A., & Bunn, H. F. (1975) *J. Clin. Invest. 56*, 201–207.
de Furia, F. G., Cerami, A., Bunn, H. F., Lee, Y. S., & Peterson, C. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3707–3710.
Klotz, I. M., & Tam, J. W. O. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1313–1315.
Perutz, M. F. (1969) *Proc. R. Soc. London, Ser. B 172*, 113.
Perutz, M. F. (1970) *Nature (London) 228*, 726–739.
Perutz, M. F. (1972) *Nature (London) 237*, 495.
Shamsuddin, M., Mason, R. G., Ritchey, J. M., Honig, G. R., & Klotz, I. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4693–4697.
Shapiro, A. L., Vinuela, E., & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.
Walder, J. A., Zaugg, R. H., Iwaoka, R. S., Watkin, W. G., & Klotz, I. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5499–5503.
Weber, K., & Osborn, A. (1969) *J. Biol. Chem. 244*, 4406–4412.
Zaugg, R. H. (1978) Ph.D. Dissertation, Northwestern University.
Zaugg, R. H., King, L. C., & Klotz, I. M. (1975) *Biochem. Biophys. Res. Commun. 64*, 1192–1198.
Zaugg, R. H., Walder, J. A., & Klotz, I. M. (1977) *J. Biol. Chem. 252*, 8542–8548.

[3] Furthermore, in the present study, bis(3,5-dibromosalicyl) sebacate, with a C_{10} bridge, was found not to cross-link either oxyhemoglobin or deoxyhemoglobin.

Purification of the Messenger Ribonucleic Acid for the Lipoprotein of the *Escherichia coli* Outer Membrane†

San-San Wang, Robert Pirtle, Irma Pirtle, Michael Small, and Masayori Inouye*

ABSTRACT: The mRNA for the lipoprotein of the *Escherichia coli* outer membrane has been purified to 85% homogeneity. The purification procedure involved phenol extraction, NaCl extraction, gel filtration on Sephadex G-100 and Sephadex G-200, and reversed-phase column chromatography on RPC-5. The purity of the final product was estimated to be 85% by analysis of the ribonuclease T_1 fingerprint of the mRNA. The purified mRNA was able to direct the synthesis of cross-reactive material with antilipoprotein serum in both the *E. coli* and the wheat germ cell-free protein-synthesizing systems. The size of the mRNA was determined to be 8.2 S from its mobility in polyacrylamide–agarose gels. During the purification, two other RNA species, similar in size to the lipoprotein mRNA, were also isolated. Their sizes were determined to be 8.7 and 9.1 S. They both were inactive in an *E. coli* cell-free protein-synthesizing system.

The lipoprotein of the *Escherichia coli* outer membrane is one of the most thoroughly investigated membrane proteins of procaryotic organisms [see a review by DiRienzo et al. (1978)]. Biosynthesis of the lipoprotein in vivo and in vitro has also been investigated [see a review by Inouye (1979)]. The mRNA for the lipoprotein has been shown to be highly stable (Hirashima & Inouye, 1973; Hirashima et al., 1973). Cross-reactive material with antilipoprotein serum was produced in the *E. coli* cell-free protein-synthesizing system (Hirashima et al., 1974), as well as in the wheat germ system (Wang et al., 1976), directed by the purified mRNA. The primary product in the *E. coli* cell-free system was found to be a precursor of the lipoprotein, prolipoprotein, which has 20 additional amino acid residues at the amino-terminal end of the lipoprotein, and the amino acid sequence of the prolipoprotein was determined (Inouye et al., 1977). The in vivo ^{32}P-labeled lipoprotein mRNA has been identified as the mRNA which codes for the lipoprotein (Takeishi et al., 1976), and the nucleotide sequence of the 5′ end of the mRNA has been determined (Pirtle et al., 1978).

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The present study describes the purification of the lipoprotein mRNA on a large scale by phenol extraction, NaCl extraction, gel filtration on Sephadex G-100 and Sephadex G-200, and reversed-phase column chromatography on RPC-5. Several other RNA species have also been isolated and characterized by gel electrophoresis and protein synthesis studies.

Materials and Methods

Bacterial Strains. *E. coli* K12 JE5519/F506 (*aro*D *lpp*$^+$ *man arg*E *lac gal rps*I *nal*A *rec*Al/F506 *aro*D$^+$ *lpp*$^+$ *man*$^+$) (Movva et al., 1978), a merodiploid strain for the structural gene of the lipoprotein (*lpp*), was used. The preincubated S-30 extract for protein synthesis was prepared from the *E. coli* Q13 strain (Nirenberg & Matthaei, 1961).

Growth Conditions. An overnight 4-L subculture of *E. coli* K12 JE5519/F506 was grown in minimal medium (M9; Inouye, 1969) supplemented with 0.2 g/L $MgSO_4 \cdot 7H_2O$, 4 g/L mannose, 2.5 mg/L thiamine, and 50 mg/L arginine. This was the starting culture for a 150-L culture grown in a New Brunswick Scientific Fermatron fermentor at 37 °C. The enriched medium for this large-scale culture contained 17.5 g/L Penassay No. 3 (Difco) and 2.3 g/L yeast extract (Difco), in addition to the minimal medium mentioned above. In the middle of the exponential phase (about 5×10^8 cells/mL), the culture was chilled to 5 °C within 20 min by using the chilling cycle of the fermentor. When the temperature of the culture reached 15 °C, chloramphenicol was added to a final concentration of 50 μg/mL. The cells were then harvested at 5 °C by using a CEPA continuous flow ultracentrifuge, which took about 4 h. About 450 g of cells was obtained and quickly frozen at −70 °C. The percentage of Man$^-$ segregants was determined by plating an appropriate dilution of the culture on eosin–methylene blue–mannose plates.

In Vitro Protein Synthesis and Immunoprecipitation. Cell-free protein synthesis was carried out as previously described (Hirashima et al., 1974), in order to measure the total amounts of proteins made by the various RNA fractions during the purification procedure. The reaction mixtures of 50 μL were incubated at 35 °C for 30 min. The incorporation of [^{35}S]methionine into hot Cl_3AcOH-insoluble material was measured in 10-μL portions of the reaction mixture.

To assay the specific activity (counts per minute of [^{35}S]lipoprotein produced per microgram of RNA), the [^{35}S]methionine-labeled cell-free products were treated with antilipoprotein serum as follows (Inouye et al., 1976). The reaction mixture (40 μL) 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 antilipoprotein serum was added to the mixture. The final mixture was incubated overnight at 4 °C. The precipitate thus formed was collected by centrifugation and washed 3 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.

One A_{260} unit is defined as that amount of material which when dissolved in 1 mL of solution will produce an absorbance of 1.0 in a 1-cm light path at 260 nm. The amount of RNA in all experiments in this paper was measured by assuming that 20 A_{260} units is equal to 1 mg of RNA.

Procedures for Fractionation of the Lipoprotein mRNA. All the procedures described below were carried out at 4 °C unless otherwise specified.

(*1*) *Phenol Extraction.* The frozen cells (450 g) were broken into small pieces. Nine hundred milliliters of extraction buffer (5 mM Tris-HCl, pH 7.4, containing 10 mM $Mg(CH_3COO)_2$ and 20 μg/mL poly(vinyl sulfate); Eastman) and 900 mL of 88% phenol (Mallinckrodt AR No. 0025) were added to the cells. This mixture was vigorously stirred for 1 h and subsequently centrifuged at 10000*g* for 10 min. The aqueous phase was collected by aspiration. The phenol phase was reextracted with 450 mL of extraction buffer. The two aqueous phases were combined and 1/10 volume of 20% $NaDodSO_4$ and 1/10 volume of 1 M sodium acetate (pH 5.2) were added to a final concentration of 2% $NaDodSO_4$ and 0.1 M sodium acetate (pH 5.2). An equal volume of fresh phenol was then added to this mixture, and the mixture was vigorously stirred at room temperature for 30 min. The aqueous phase was collected by centrifugation as above, and 2.5 volumes of 95% ethanol (Mallinckrodt AR No. 7019) was added to the aqueous phase. The RNA was precipitated at −20 °C overnight and collected by centrifugation at 10000*g* for 30 min.

(*2*) *NaCl Extraction.* The crude RNA precipitate was dispersed in 200 mL of 1 M NaCl (Zubay, 1962), and the mixture was vigorously stirred for 1 h to dissolve the smaller RNA fractions, leaving the 16S and 23S rRNAs as precipitates. The ribosomal RNA precipitate was removed by centrifugation at 10000*g* for 10 min. This precipitate was reextracted with 100 mL of 1 M NaCl and subsequently centrifuged. The combined soluble RNA fractions were precipitated by the addition of 2.5 volumes of ethanol and kept at −20 °C overnight.

(*3*) *Gel Filtration on Sephadex G-100.* The RNA precipitate from the NaCl extraction was collected by centrifugation at 12000*g* for 30 min and dissolved in 60 mL of 10 mM NaCl, 10 mM sodium acetate (pH 5.2), 10 mM EDTA, and 4 M urea. One-third of this solution was applied to a 4.5 × 86 cm Sephadex G-100 column (40–120 μm; Pharmacia) equilibrated with 0.1 M NaCl. The elution was carried out with 0.1 M NaCl. The fractions were analyzed spectrophotometrically at 260 nm. Appropriate fractions were pooled and assayed for mRNA activity in a cell-free system.

(*4*) *Gel Filtration on Sephadex G-200.* The fractions from the Sephadex G-100 columns which had the highest mRNA activity were ethanol-precipitated as described above. The RNA pellets were redissolved in 12 mL of 10 mM NaCl, 10 mM sodium acetate (pH 5.2), 10 mM EDTA, and 4 M urea. Portions of this solution were applied to a number of 2 × 68 cm Sephadex G-200 columns in parallel (10–40 μm; Pharmacia Superfine) equilibrated with 0.1 M KCl. The elution was carried out with 0.1 M KCl at 4 °C. Fractions were analyzed by absorbance readings at 260 nm. The ability of the RNA fractions to stimulate the incorporation of [^{35}S]-methionine into Cl_3AcOH-insoluble material was examined in the cell-free system. Appropriate fractions from these columns were usually pooled and run on smaller Sephadex G-200 columns under the same conditions for improved resolution. The Sephadex G-200 fractions containing the lipoprotein mRNA were precipitated with ethanol and centrifuged as above.

(*5*) *Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoresis was performed according to the method of Peacock & Dingman (1967).

Electrophoresis was carried out at 4 °C, using TEB buffer (pH 8.3), which consisted of 10.8 g of Tris, 0.93 g of Na_2EDTA, and 5.5 g of boric acid per L. The RNA samples were dissolved in 20 μL (or 200 μL for preparative gels) of water and mixed with 5 μL (or 50 μL for preparative gels) of TEB buffer (pH 8.3) containing 40% sucrose, 0.1 M EDTA, 0.1 M NaCl, and 0.1% bromphenol blue (BPB). Before application, the samples were heated at 90 °C for 2 min and

Table I: Summary of Purification of Lipoprotein mRNA

purification step	total RNA (mg)	total act. (cpm)[a]	sp act. [(cpm × 10^{-3})/mg of RNA][b]
phenol extraction	1780	4.0×10^6 (100)	2.2 (1)
1 M NaCl fractionation	1250	3.4×10^6 (85)	2.7 (1.2)
Sephadex G-100 column	505	3.2×10^6 (80)	6.3 (2.9)
Sephadex G-200 column	49	2.9×10^6 (72)	59.2 (27)
band 4 from gel	2.6	1.6×10^6 (40)	615 (280)
RPC-5 column (Figure 5A)	0.12	1.2×10^5 (3)	1000 (454)

[a] The total activity is the product of the amount of RNA and the specific activity of the RNA. The percent yield is indicated in parentheses. [b] The specific activity is defined under Materials and Methods. The numbers in parentheses represent purification-fold.

quickly cooled. The RNA samples were then fractionated on 5% polyacrylamide gels for 2.5 h at 150 V.

The positions of the RNA bands in preparative gels were determined by staining a small vertical strip from one side of the gel. A thin slice corresponding to each RNA band was excised from the slab gel with the aid of the stained gel strip. The gel slice (about 0.3 × 12 × 0.3 cm) was frozen at −70 °C and crushed. These small gel pieces were homogenized in a glass homogenizer with 5 mL of 0.4 M NaCl. The mixture was then transferred into a small beaker and stirred overnight at 5 °C. The supernatant was collected by centrifugation at 12000*g* for 10 min. The gel pellet was reextracted again with 2 mL of 0.4 M NaCl for 2 h. This was centrifuged as above. The two resulting supernatants were combined and filtered through a 0.45-μm Millipore filter to remove fine gel particles. The filtrate was lyophilized to half the original volume, and then 1/10 volume of 20% potassium acetate and 2.5 volumes of 95% ethanol were added to the filtrate. The mixture was kept at −20 °C overnight. The RNA precipitate thus formed was collected by centrifugation at 12000*g* for 30 min and dissolved in distilled water. Ethanol precipitation was repeated, and the final RNA precipitate was dissolved in 0.5 mL of water. The specific activities of the RNA extracted from these bands were assayed in the cell-free system.

Other Polyacrylamide Gels. For molecular weight determinations of RNA samples, 3% polyacrylamide–0.5% agarose gels were run according to the method of Peacock & Dingman (1968). Electrophoresis was carried out on 0.15 × 15 × 11 cm gels in TEB buffer (pH 8.3) at 100 V until the bromphenol blue dye marker was about 1 cm from the bottom of the gel. Gels were stained in 0.2% methylene blue in 1 N acetic acid for 10 min and then destained with running water for 2 h. In order to assess the purity and quality of the RNA samples during the various stages of purification, 5% polyacrylamide gels (0.15 × 15 × 11 cm) were done (Peacock & Dingman, 1967) for 2.5 h at 150 V. These gels were stained and destained as above.

$NaDodSO_4$ slab gel electrophoresis of the protein products from cell-free synthesis was carried out by using 17.5% polyacrylamide gels according to the method of Anderson et al. (1973). After gel electrophoresis, the gel was dried by using a Hoefer slab gel dryer. For autofluorography (Bonner & Laskey, 1974) Kodak BB-5 X-ray film was used.

Reversed-Phase Chromatography. The RPC-5 (Plaskon) reversed-phase support (a gift from Dr. Bernard Dudock) was coated with Adogen 464 (trialkylmethylammonium chloride) by method C of Pearson et al. (1971). High-pressure chromatography columns were from Glenco (Houston, TX). A Milton Roy minipump with a maximum pressure rating of 5000 psig was used to generate the flow rate. The mRNA-containing fractions from the Sephadex G-200 columns were precipitated as described above, and the residual ethanol was removed by lyophilization over P_2O_5. The lyophilized sample was dissolved in 300–400 μL of TM buffer containing 50 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, and 0.4 M NaCl (Kelmers et al., 1974) and applied to a 0.9 × 90 cm RPC-5 column equilibrated in the same buffer. A linear 0.4–1.1 M NaCl gradient was used for elution. To measure the activity of the mRNA containing fractions in the cell-free system, a portion of appropriate fractions containing 1 μg of RNA was removed, mixed with 10 μg of *E. coli* B carrier tRNA, and precipitated twice by the addition of 1/10 volume of 20% potassium acetate (pH 5.2) and 2.5 volumes of ethanol. The fractions exhibiting the highest protein synthesis activity were pooled and precipitated.

For further purification, the lipoprotein mRNA fraction was applied to a 0.6 × 60 cm RPC-5 column and run under essentially the same conditions as the larger column.

Estimation of Purity of Lipoprotein mRNA by Two-Dimensional Electrophoresis–Homochromatography. The fragments produced by ribonuclease T_1 and alkaline phosphatase digestion (Brownlee, 1972) of the highly purified mRNA from the RPC-5 column were labeled at their 5′ termini with [γ-^{32}P]ATP and polynucleotide kinase (Simsek et al., 1973; Gillum et al., 1975) and "fingerprinted" by two-dimensional electrophoresis–homochromatography as described elsewhere (Takeishi et al., 1976). Comparison of the radioactivity of oligonucleotides derived from the lipoprotein mRNA and that of fragments of comparable chain length derived from impurities from other RNAs was used to estimate the purity of the lipoprotein mRNA.

Results

Purification Procedures. About 1.8 g of crude RNA was isolated by phenol deproteinization and ethanol precipitation. During the extraction of the *E. coli* cells in aqueous phenol, tRNA, 5S RNA, and a number of smaller RNAs are leached out through the cell wall, while DNA and a good deal of the high molecular weight RNAs are retained inside (Monier et al., 1960). Thus, the lipoprotein mRNA is actually extracted from the cell suspension by using preparation procedures previously used for tRNA (Zubay, 1962).

The second stage of extraction (NaCl extraction) removed a considerable amount of ribosomal RNAs and other RNAs of large molecular weights which would cause loading problems (due to viscosity) and poor resolution during gel filtration on the Sephadex G-100 columns. About 1.3 g of crude RNA was recovered after the NaCl extraction (Table I), but the specific activity for the lipoprotein mRNA did not increase significantly, as measured in the cell-free system.

The next stages of purification involved the fractionation of the NaCl-extracted RNA by gel filtration on Sephadex G-100, running the NaCl-extracted RNA on several columns in parallel. Figure 1 shows the elution profile of a typical Sephadex G-100 column. The three major peaks of absorbance at 260 nm were pooled into portions P1, P2, and P3, as shown in Figure 1. It was found that only the P1 fraction

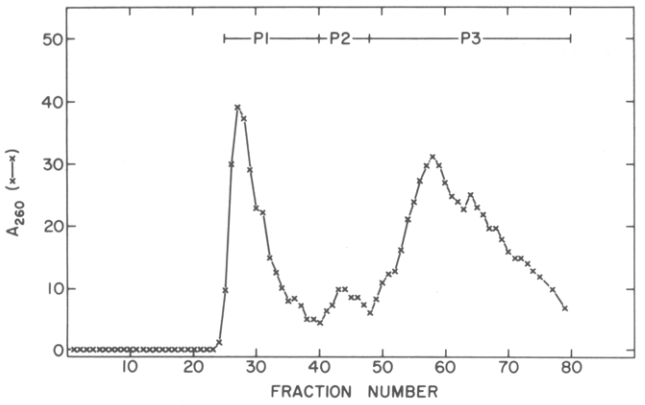

FIGURE 1: Fractionation of RNA by gel filtration on Sephadex G-100. A portion of the ethanol-precipitated supernatant obtained by NaCl fractionation, containing about 8000 A_{260} units (400 mg), was dissolved in 10 mM sodium acetate (pH 5.2), 10 mM NaCl, 10 mM Na_2EDTA, and 4 M urea and applied to a Sephadex G-100 column (4.5 × 86 cm) equilibrated in 0.1 M NaCl. Fractions of about 12 mL were collected at a flow rate of 30 mL/h. The absorbance (at 260 nm) of 1/100 dilutions of the fractions was measured, and the A_{260} reading per milliliter of the fractions was calculated. The two major fractions (indicated by the solid lines as P1 and P2) were pooled and ethanol-precipitated. The P3 fractions were discarded.

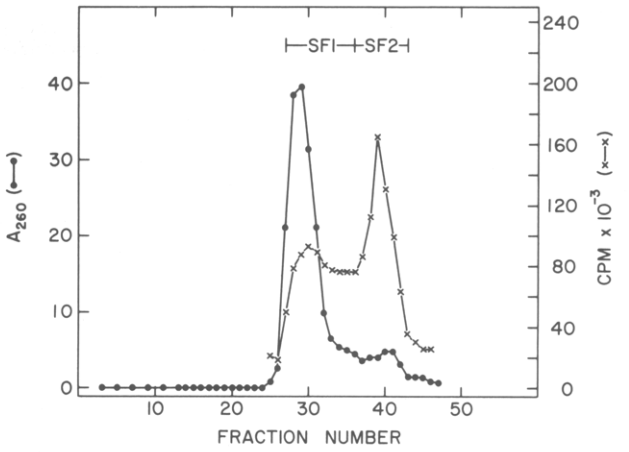

FIGURE 2: Fractionation of RNA by gel filtration on Sephadex G-200. About 750 A_{260} units (37.5 mg) of the ethanol-precipitated P1 fractions from one of the Sephadex G-100 columns was dissolved in 3.0 mL of 10 mM sodium acetate (pH 5.2), 10 mM NaCl, 10 mM Na_2EDTA, and 4 M urea and applied to a Sephadex G-200 column (2 × 68 cm). Fractions of 3.5 mL were collected at a flow rate of 5 mL/h. The absorbances (at 260 nm) of 1/100 dilutions of the fractions were measured, and the A_{260} reading per milliliter of the fractions was calculated. For assaying the mRNA activity, 20 μL of the fractions was used to measure the radioactivity of [^{35}S]methionine incorporated into Cl_3AcOH-insoluble material in a cell-free system. Fractions SF-1 and fractions SF-2 were pooled and ethanol-precipitated, as indicated by the solid lines.

had mRNA activity in the cell-free system, indicative of the presence of mRNA (data not shown). When the P2 and P3 fractions were further analyzed by 5% polyacrylamide gels, the P2 fraction mainly contained 5S and 6S RNAs and the P3 fraction contained 4S RNA (data not shown). In this particular gel filtration on Sephadex G-100, the recovery of A_{260}-absorbing material was essentially 100%. The percentages of the P1, P2, and P3 fractions were 36, 10, and 54%, respectively. Thus, RNAs of smaller molecular weights were separated from the P1 fraction which contained mRNA as assayed by cell-free synthesis, and the specific activity of the mRNA was greatly enhanced, without any loss of mRNA at this stage of purification (Table I).

The RNA from the P1 fractions was ethanol-precipitated and dissolved as described under Materials and Methods for application to Sephadex G-200 columns for further fractionation. A typical fractionation profile of the P1 fraction on a Sephadex G-200 column is shown in Figure 2. About 85% of the P1 fraction applied to the Sephadex G-200 column appeared in the void volume (SF-1), and about 15% of the total RNA was recovered as a broad peak (SF-2) after the void volume. When the ability of the RNA fractions to stimulate the incorporation of [^{35}S]methionine into the hot trichloroacetic acid insoluble material in the cell-free system was examined (see Figure 2), it was found that the RNA from the SF-2 fraction had much higher mRNA activity than the RNA from the SF-1 fraction. The RNA fractions from the Sephadex G-200 columns were pooled into two portions, SF-1 and SF-2, as shown in Figure 2. The cell-free products directed by these fractions were examined immunologically with antilipoprotein serum. About 20% of the total products directed by the SF-2 fraction was recovered by immunoprecipitation, in contrast to 3% by the SF-1 fractions. Therefore, it was concluded that the SF-2 fractions contained the lipoprotein mRNA. The specific activity as measured by the cell-free protein synthesis system increased about 10-fold over the previous step (Table I).

When the SF-1 and SF-2 fractions from the Sephadex G-200 columns were analyzed by polyacrylamide gel electrophoresis, followed by staining with methylene blue, they contained a number of distinct bands, as shown in Figure 3. In order to examine which band in the SF-2 RNA fractions

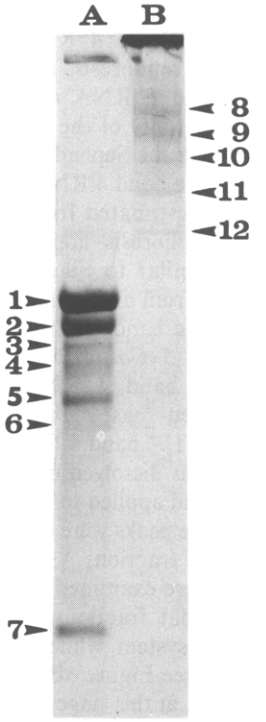

FIGURE 3: Slab gel electrophoresis of RNA fractions SF-1 and SF-2 from gel filtration on Sephadex G-200. The gel was prepared by layering a 3% stacking gel (0.15 × 11 × 2 cm) on top of a 5% gel (0.15 × 11 × 10 cm). Electrophoresis was carried out in TEB buffer (pH 8.3) at 150 V for 2.5 h at room temperature. The RNA samples were dissolved in 20 μL of TEB buffer (pH 8.3) containing 40% sucrose, 0.1 M EDTA, 0.1 M NaCl, and 0.1% BPB. The samples are as follows: (A) 16 μg of SF-2; (B) 60 μg of SF-1.

corresponds to the lipoprotein mRNA, we extracted RNAs from bands 1–5 of the gel, and the mRNA activity of each band was examined. It was found that band 4 had the highest mRNA activity, as shown in Figure 4.

In order to preparatively purify the RNA in band 4, we applied 1.5 mg of the SF-2 RNA fraction to a 5% poly-

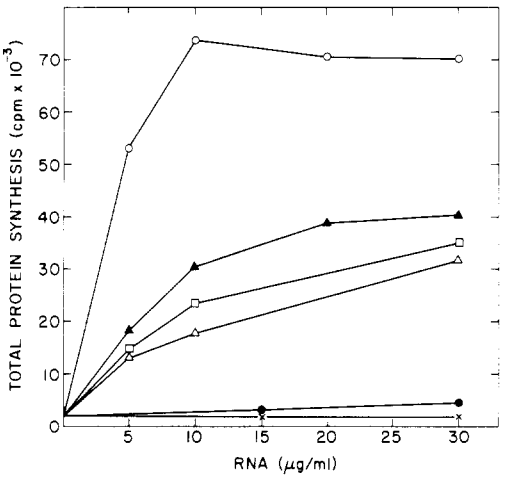

FIGURE 4: Messenger RNA activity of RNA fractions from SF-2. About 300 μg of the SF-2 fraction was applied to a 5% polyacrylamide gel (0.3 × 6 × 11 cm), and electrophoresis was carried out at 150 V for 2.5 h at 4 °C. Bands corresponding to bands 1–5 in Figure 3 were then extracted from the gel as described under Materials and Methods. Incorporation of [^{35}S]methionine into hot Cl_3AcOH-insoluble material was measured in the *E. coli* cell-free system, directed by RNA from the following sources: (▲) SF-2; (×) band 1; (●) band 2; (□) band 3; (○) band 4; (△) band 5.

acrylamide gel (0.3 × 15 × 11 cm), and the electrophoresis was carried out as described under Materials and Methods. About 0.08 mg of band 4 RNA was recovered from the gel. When the purity of the band 4 RNA was examined by polyacrylamide gel electrophoresis, it still contained small amounts of bands 3 and 5 RNA as shown in Figure 3. However, the specific activity of the lipoprotein mRNA increased about 10-fold over the Sephadex G-200 step (data not shown). The purity of the band 4 RNA from the 5% gel was on the order of 30% as estimated from examination of the two-dimensional electrophoresis–homochromatography fingerprint (not shown, similar to Figure 8a) of the in vitro [5-^{32}P]oligonucleotides from a ribonuclease T_1 and alkaline phosphatase digest of the band 4 RNA.

Purification by RPC-5 Column Chromatography. A further purification of band 4 RNA from another 5% acrylamide gel by reversed-phase chromatography on RPC-5 is shown in Figure 5A. The band 4 RNA fraction extracted from the gel (180 μg) was dissolved in 300 μL of TM buffer (Kelmers et al., 1974) and applied to the RPC-5 column. As shown in Figure 5A, three peaks were eluted from the column. The mRNA activities of fractions A–C (as indicated by the arrows in Figure 5A) were examined in the cell-free system. The results indicated that fraction B could direct protein synthesis in the cell-free system, while fractions A and C had little mRNA activities (see Figure 5B). The specific activity of the lipoprotein mRNA at this stage increased about twofold over the previous step. To examine the purity of fraction B RNA, it was digested with ribonuclease T_1 and fingerprinted by two-dimensional electrophoresis–homochromatography. The pattern was almost identical with the one previously published for in vivo ^{32}P-labeled lipoprotein mRNA, which was purified by completely different procedures (Takeishi et al., 1976). This supports our conclusion that fraction B RNA is the mRNA for the lipoprotein. From this fingerprint, the purity of the lipoprotein mRNA by these procedures was determined to be 73% (data not shown). At this stage, the mRNA had been purified about 450-fold over the crude extract (Table I). One can obtain about 120 μg of the RNA fraction from 450 g of cells, with about 3% recovery of the lipoprotein mRNA.

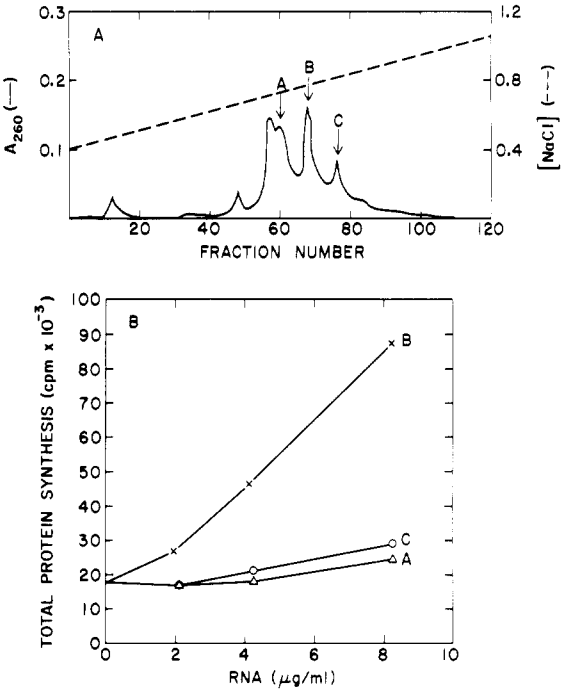

FIGURE 5: Reversed-phase chromatography of band 4 RNA from polyacrylamide gel electrophoresis. (A) About 180 μg of band 4 from the polyacrylamide gel electrophoresis (Figure 3) was dissolved in 300 μL of TM buffer (Kelmers et al., 1974) containing 50 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, and 0.4 M NaCl and applied to a RPC-5 column (0.6 × 60 cm) equilibrated with TM buffer. A linear 0.4–1.1 M NaCl gradient was used for the elution at room temperature. Fractions of 1 mL were collected at a flow rate of 0.5 mL/min. The three fractions (A–C, as indicated by the arrows) were pooled and analyzed for mRNA activity in the cell-free system. The RNA from the peak tube from fraction B was fingerprinted by two-dimensional electrophoresis–homochromatography. (B) Messenger RNA activity of the RNA fractions from the reversed-phase column chromatography. The RNA fractions A–C from Figure 5A were pooled and dialyzed against 1 L of distilled water overnight. Incorporation of [^{3}H]leucine into hot Cl_3AcOH-insoluble material in the cell-free system directed by various amounts of each RNA fraction was measured: (△) fraction A; (×) fraction B; (○) fraction C.

Isolating large amounts of mRNA from a polyacrylamide gel, followed by chromatography on a small RPC-5 column, proved to be laborious, since the amount of material from the SF-2 fraction of the Sephadex G-200 column which could be applied to the gel was small in comparison with the amount of SF-2 RNA which had to be processed. We attempted to develop a new purification procedure using only reversed-phase chromatography on RPC-5 after the Sephadex G-200 columns. In a typical experiment, about 100 A_{260} units (5 mg) of the mRNA-containing fraction (SF-2) was dissolved in TM buffer and applied to a 0.9 × 90 cm RPC-5 column. As shown in Figure 6B, the SF-2 fraction was separated into a number of peaks. In order to measure the mRNA activity, an aliquot containing 1 μg of RNA was removed from each fraction and mixed with 1 μL of 10 mg/mL *E. coli* B carrier tRNA, and the RNA was then precipitated by the addition of 1/10 volume of 20% potassium acetate (pH 5.2) and 2.5 volumes of ethanol. Thus, the mRNA activity of each fraction was obtained and its profile is shown in Figure 6A. It is clearly seen that fractions 114–122 have the highest specific activities. These RNA fractions eluted at approximately the same NaCl concentration as in vivo ^{32}P-labeled lipoprotein mRNA run on a smaller column under the same gradient conditions and about the same NaCl concentration as fraction B in Figure 5A. The tubes in this fraction were pooled, ethanol-precip-

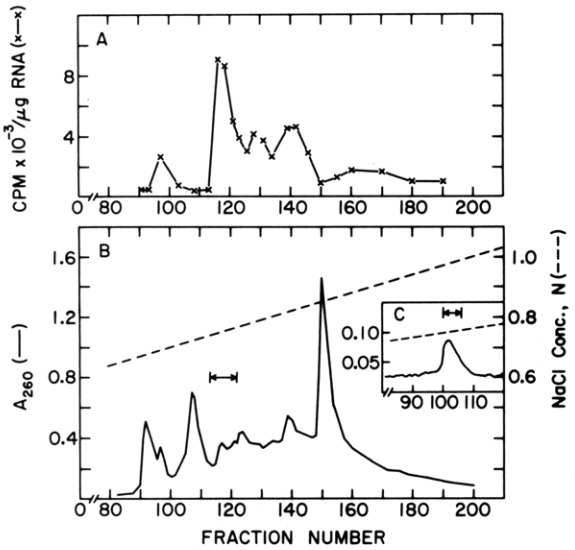

FIGURE 6: Isolation of lipoprotein mRNA by reversed-phase chromatography on RPC-5. About 80 A_{260} units (4 mg) of the SF-2 fraction from one of the Sephadex G-200 columns was dissolved in 250 μL of TM buffer (pH 7.3) and applied to a RPC-5 column (0.9 × 90 cm) equilibrated in TM buffer and eluted by a linear 0.4–1.1 M NaCl gradient at room temperature as shown in (B). Fractions of 1.7 mL were collected at a flow rate of 30 mL/h. Cell-free protein synthesis was carried out in 25 μL of the reaction mixture at the RNA concentration of 2 μg/mL for each fraction. The hot Cl_3AcOH-insoluble radioactive material was then plotted in (A). The fractions were pooled as indicated by the arrow in (B). The pooled fractions were further purified on a smaller (0.6 × 60 cm) RPC-5 column run under the same conditions as shown in the inset, (C). The fractions were pooled as indicated by the arrow in (C).

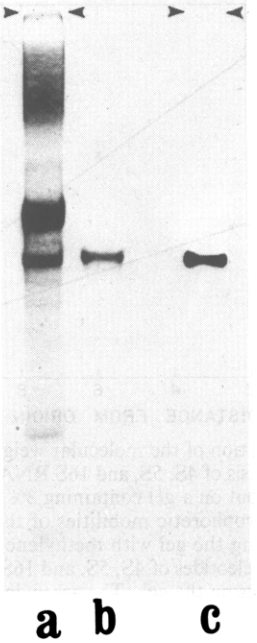

FIGURE 7: Fractionation of lipoprotein mRNA on 5% polyacrylamide gel. About 16 μg of the SF-2 fraction from a Sephadex G-200 column was applied in slot a of a 5% polyacrylamide gel. About 8 μg of nonradioactive band 4 RNA, extracted from a polyacrylamide slab gel made of a 3% stacking gel (0.3 × 13 × 2 cm) layered on top of a 5% gel (0.3 × 13 × 19 cm), was applied in slot b. In vivo ^{32}P-labeled lipoprotein mRNA, isolated as described by Takeishi et al. (1976), was applied in slot c. Electrophoresis was carried out in TEB buffer (pH 8.3) at 150 V for 2.5 h at room temperature. Lanes a and b were sliced from the gel and stained with methylene blue while lane c was placed in autoradiography using Kodak BB-5 X-ray film. The stained and dried portion of the gel was matched with the corresponding autoradiogram.

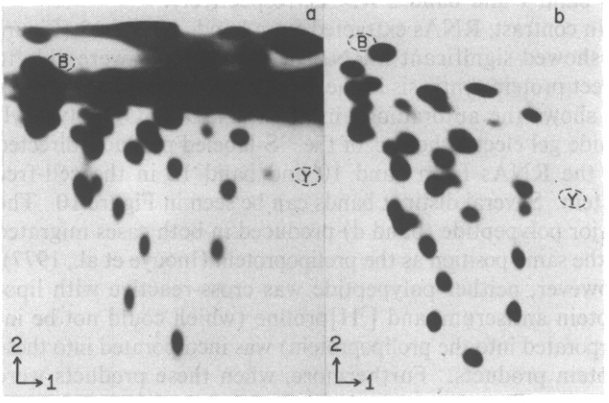

FIGURE 8: Identification and estimation of purity of the lipoprotein mRNA. A ribonuclease T_1 digest of the mRNA from the RPC-5 column shown in Figure 6C was labeled with ^{32}P and fractionated by two-dimensional electrophoresis–homochromatography as indicated under Materials and Methods. First dimension: electrophoresis on cellulose acetate in 5% acetic acid, 5 mM EDTA, and 7 M urea (pH 3.5). Second dimension: homochromatography on DEAE-cellulose at pH 7 and 60 °C (Sanger et al., 1973). (a) Autoradiogram of the fingerprint of [5′-^{32}P]oligonucleotides from a ribonuclease T_1 digest of the mRNA; (b) autoradiogram of the fingerprint of oligonucleotides from a ribonuclease T_1 digest of in vivo ^{32}P-labeled lipoprotein mRNA, isolated as described by Takeishi et al. (1976). The purity of the mRNA was estimated as described by Takeishi et al. (1976).

itated, and applied to a smaller (0.6 × 60 cm) RPC-5 column for further purification. As shown in Figure 6C, a major peak appeared at about the same NaCl concentration as shown in Figure 6B, and the fractions in this peak were shown to have the highest mRNA activity (data not shown).

Identification of the Lipoprotein mRNA. The RNA fraction thus purified was deduced to be the lipoprotein mRNA from the following reasons. (a) The RNA could direct the production of cross-reactive material against antilipoprotein serum in the *E. coli* cell-free protein synthesizing system and had the highest specific activity among any RNA fractions in the purification procedure (Table I). (b) Figure 7 indicates that the nonradioactive band 4 RNA has the same electrophoretic mobility as the in vivo ^{32}P-labeled mRNA isolated as described previously (Takeishi et al., 1976). Furthermore, peak B of the RPC-5 column in Figure 5A has the same mobility as the band 4 RNA, as well as in vivo ^{32}P-labeled mRNA. (c) The most critical identification was carried out by fingerprinting by two-dimensional electrophoresis–homochromatography of ribonuclease T_1 fragments of the purified RNA, as shown in Figure 8a. This autoradiogram is essentially identical with that obtained for the in vivo ^{32}P-labeled lipoprotein mRNA, shown in Figure 8b. The in vivo ^{32}P-labeled lipoprotein mRNA was identified as the lipoprotein mRNA by nucleotide sequence analysis (Takeishi et al., 1976). The purity of the final mRNA fraction from the RPC-5 column (Figure 6C) was estimated to be 85% by assuming that the radioactivity of minor spots was due to contaminating oligonucleotides. It should be noted that minor changes occurred in the mobilities of certain fragments such as the slight upward shifts of some fragments on the right side of the in vitro labeled ribonuclease T_1 fingerprint (Figure 8a) as compared to the in vivo labeled ribonuclease T_1 fingerprint (Figure 8b). This perhaps can be attributed to the change from a 3′-phosphate group in the in vivo labeled fragments to a 5′-phosphate group in the in vitro labeled fragments. The streaking at the top of Figure 8a is due to the presence of the [γ-^{32}P]ATP-derived artifacts.

The size of the lipoprotein mRNA was estimated to be about 8.2 S or about 350 nucleotides by comparing its electrophoretic

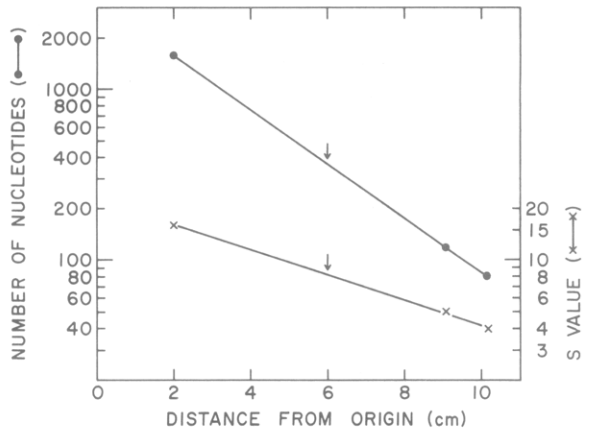

FIGURE 9: Determination of the molecular weight of the lipoprotein mRNA. Electrophoresis of 4S, 5S, and 16S RNAs and the lipoprotein mRNA was carried out on a gel containing 3% polyacrylamide and 0.5% agarose. Electrophoretic mobilities of these RNAs were determined after staining the gel with methylene blue. The S values and the number of nucleotides of 4S, 5S, and 16S RNAs were plotted against their mobilities on the gel. The arrow in the figure indicated the mobility of the lipoprotein mRNA on the gel.

mobility with that of the electrophoretic mobilities of *E. coli* 4S, 5S, and 16S RNAs on a 3% polyacrylamide–0.5% agarose gel and plotting electrophoretic mobility vs. S value, as shown in Figure 9.

Other RNA Fractions. The two major RNA fractions from bands 1 and 2 of the SF-2 RNA from the Sephadex G-200 column (see Figure 3) were also isolated as single bands by gel electrophoresis (data not shown). However, neither of them had mRNA activity in the *E. coli* cell-free system. Their sizes were estimated by polyacrylamide–agarose gel electrophoresis to be about 9.1 S (440 nucleotides) and 8.7 S (400 nucleotides) for band 1 and band 2 RNAs, respectively.

In contrast, RNAs extracted from bands 10 and 12 (Figure 3) showed significant mRNA activity as they were able to direct protein synthesis in the *E. coli* cell-free system. Figure 10 shows the autoradiogram of the $NaDodSO_4$–polyacrylamide gel electrophoresis of the ^{35}S-labeled products directed by the RNAs from band 10 and band 12 in the cell-free system. Several distinct bands can be seen in Figure 10. The major polypeptide (band d) produced in both cases migrated at the same position as the prolipoprotein (Inouye et al., 1977). However, neither polypeptide was cross-reactive with lipoprotein antiserum, and [^3H]proline (which could not be incorporated into the prolipoprotein) was incorporated into these protein products. Furthermore, when these products were analyzed in a different $NaDodSO_4$ gel electrophoresis system using 10 mM sodium phosphate buffer (pH 7.1) containing 0.5% $NaDodSO_4$ (Inouye & Pardee, 1970), both products migrated faster than the prolipoprotein (data not shown). These results indicate that the RNAs in bands 10 and 12 can direct the production of polypeptides which are distinctly different from the prolipoprotein. It is not known at present if the polypeptide in band d (directed by band 10 RNA) is the same as that directed by band 12 RNA. The band 10 RNA fraction appears to contain several mRNAs since several other protein products (bands a–c in Figure 10) are also produced. The apparent molecular weights of these polypeptides were estimated to be 18 000, 14 500, 11 000, and 10 000 for bands a, b, c, and d, respectively.

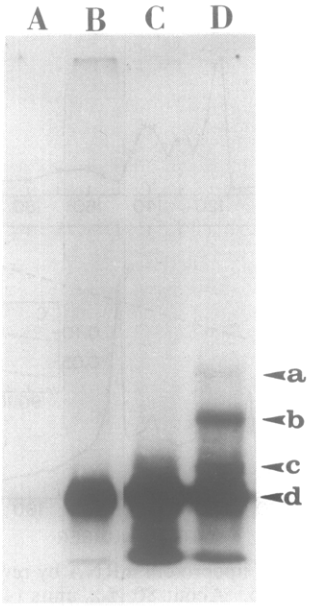

FIGURE 10: $NaDodSO_4$–polyacrylamide gel electrophoresis of cell-free products obtained by protein synthesis. Bands 10 and 12 from the gel in Figure 3 were used to synthesize protein products. After incubation at 35 °C for 30 min, 2 mL of cold 5% trichloroacetic acid was added to the reaction mixture (50 μL), and the mixture was incubated in a boiling water bath for 15 min. The Cl_3AcOH-insoluble material was then washed twice with 2 mL of ether–alcohol (1:1) and twice with 2 mL of ether. The pellet was dried with an air stream and dissolved in 0.1 mL of 10 mM sodium phosphate buffer (pH 7.1) containing 1% (w/v) $NaDodSO_4$, 10% (v/v) glycerol, and 1% (v/v) β-mercaptoethanol (Inouye & Pardee, 1970). The solution was then incubated at 70 °C for 20 min. Twenty microliters of each sample was applied to the gel. Gel electrophoresis was carried out on a 17.5% acrylamide gel (Anderson et al., 1973). (A) Control (minus mRNA); (B) prolipoprotein; (C) cell-free products directed by band 12 RNA; (D) cell-free products directed by band 10 RNA.

Discussion

The lipoprotein mRNA is the first biologically active mRNA purified from *E. coli* cells. The mRNA appears to be monocistronic since 234 nucleotides are required to code for prolipoprotein and since the mRNA has recently been found to be 322 nucleotides in length (R. Pirtle, I. Pirtle, and M. Inouye, unpublished experiments). The lipoprotein mRNA also is the first bacterial mRNA to be isolated by reversed-phase column chromatography, and this technique could prove useful for the isolation of other abundant bacterial mRNAs, since the absence of a poly(A) tail does not complicate the resolution profiles.

The nonradioactive RNAs isolated in this paper (bands 1–12 in Figure 3 and the various peaks from the RPC-5 column in Figure 7b) may correspond to some in vivo ^{32}P-labeled RNAs observed by Ikemura & Dahlberg (1973) and Takeishi et al. (1976) and 10S RNAs recently identified by Lee et al. (1978). In particular, the RNA from band VIII of Ikemura & Dahlberg (1973) could very well be the lipoprotein mRNA, judging from the relative mobilities in the gels. Band IX in their paper can be estimated to be about 9 S on the basis of the relative mobility in the gel, corresponding to 300–400 nucleotides in chain length. Since band 1 and band 2 RNAs can be calculated to have about the same S values, it is possible that one of these RNAs could correspond to that of band IX of Ikemura & Dahlberg (1973). Furthermore, Lee et al. (1978) have made an estimate of 10 S for a band containing two RNAs, roughly estimated to be 500 nucleotides in length. It is possible that bands 1 and 2 could correspond to their 10S RNA band, since the S values were measured in entirely different polyacrylamide gel systems. Furthermore, band VI of Takeishi et al. (1976) also appears to have about the same electrophoretic mobility as band 1 and band 2 RNAs in the

5% gel system. These RNAs seem to be abundant and stable like the lipoprotein mRNA, but their biological function is unknown.

The sequence of the extension peptide at the N-terminal end of the prolipoprotein has been determined by using in vitro cell-free products directed by the purified mRNA (Inouye et al., 1977). This also indicated that the purified mRNA has the same biological activity as in the cell. The fact that the lipoprotein mRNA can be isolated in reasonably good purity and in a reasonable quantity from both gels and RPC-5 columns further indicates that the lipoprotein mRNA is an abundant and stable *E. coli* mRNA. The stability of the mRNA was demonstrated by Hirashima et al. (1973), who showed the functional half-life of the lipoprotein mRNA to be 11.5 min, more stable than other *E. coli* mRNAs with half-lives of 2–4 min. Since the lipoprotein is the most abundant protein in the *E. coli* cell, it is possible that the lipoprotein mRNA is one of the most abundant mRNAs in *E. coli*. Thus, the lipoprotein mRNA provides an ideal model system for the study of the mechanism of biosynthesis and assembly of membrane proteins.

References

Anderson, C., Baum, P., & Gesteland, R. (1973) *J. Virol. 12*, 241–252.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Brownlee, G. G. (1972) in *Determination of Sequences in RNA*, Elsevier, New York.

DiRienzo, J. M., Nakamura, K., & Inouye, M. (1978) *Annu. Rev. Biochem. 47*, 481–532.

Gillum, A. M., Urquhart, N., Smith, M., & RajBhandary, U. L. (1975) *Cell 6*, 395–405.

Hirashima, A., & Inouye, M. (1973) *Nature (London) 242*, 405–407.

Hirashima, A., Childs, G., & Inouye, M. (1973) *J. Mol. Biol. 79*, 373–389.

Hirashima, A., Wang, S. S., & Inouye, M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4149–4153.

Ikemura, T., & Dahlberg, J. E. (1973) *J. Biol. Chem. 248*, 5024–5032.

Inouye, M. (1969) *J. Bacteriol. 99*, 842–850.

Inouye, M. (1979) *Biomembranes 10* (in press).

Inouye, M., & Pardee, A. B. (1970) *J. Biol. Chem. 245*, 5813–5819.

Inouye, S., Takeishi, K., Lee, N., DeMartini, M., Hirashima, A., & Inouye, M. (1976) *J. Bacteriol. 121*, 555–563.

Inouye, S., Wang, S. S., Sekizawa, J., Halegoua, S., & Inouye, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1004–1008.

Kelmers, A., Heatherly, D., & Egan, B. (1974) *Methods Enzymol. 29*, 483–486.

Lee, S. Y., Bailey, S. C., & Apirion, D. (1978) *J. Bacteriol. 133*, 1015–1023.

Monier, R., Stephenson, M. L., & Zamecnik, P. C. (1960) *Biochim. Biophys. Acta 43*, 1–8.

Movva, N. R., Katz, E., Asdourian, P. L., Hirota, Y., & Inouye, M. (1978) *J. Bacteriol. 133*, 81–84.

Nirenberg, M. W., & Matthaei, J. H. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1588–1602.

Peacock, A. C., & Dingman, C. W. (1967) *Biochemistry 6*, 1818–1827.

Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.

Pearson, R., Weiss, J., & Kelmers, A. (1971) *Biochim. Biophys. Acta 228*, 770–774.

Pirtle, R., Pirtle, I., & Inouye, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2190–2194.

Sanger, F., Donelson, J., Coulson, A., Kossel, H., & Fischer, D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1209–1213.

Simsek, M., Ziegenmeyer, J., Heckman, J., & RajBhandary, U. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1041–1045.

Takeishi, K., Yasumura, M., Pirtle, R., & Inouye, M. (1976) *J. Biol. Chem. 251*, 6259–6266.

Wang, S. S., Marcu, K. B., & Inouye, M. (1976) *Biochem. Biophys. Res. Commun. 68*, 1194–1200.

Zubay, G. (1962) *J. Mol. Biol. 4*, 347–356.