Fox, J. B., Nicholas, R. A., Ackerman, S. A., & Swift, C. E. (1974) *Biochemistry 13*, 5178-5186.
Frost, A. A., & Pearson, R. G. (1953) Kinetics and Mechanism, pp 173-177, John Wiley & Sons, New York.
Glass, J., & Nunez, M. T. (1986) *J. Biol. Chem. 261*, 8298-8302.
Honkakoski, P. J., & Hassinen, I. E. (1986) *Biochem. J. 237*, 927-930.
Iacopetta, B. J., & Morgan, E. H. (1983) *J. Biol. Chem. 258*, 9108-9115.
Li, C.-Y., Hamazaki, S., Watkins, J. A., Altazan, J. D., & Glass, J. (1991a) *FASEB J. 5*, A749.
Li, C.-Y., Watkins, J. A., & Glass, J. (1991b) *Blood 78*, 87a.
Nunez, M. T., & Glass, J. (1985) *J. Biol. Chem. 260*, 14707-14711.
Nunez, M. T., Cole, E. S., & Glass, J. (1983) *J. Biol. Chem. 258*, 1146-1151.
Nunez, M. T., Pinto, I., & Glass, J. (1989) *J. Membr. Biol. 107*, 129-135.
Nunez, M. T., Gaete, V., Watkins, J. A., Glass, J. (1990) *J. Biol. Chem. 265*, 6688-6692.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 560-590, John Wiley & Sons, New York.
Sun, I. L., Crane, F. L., Grebing, C., & Low, H. (1984) *J. Bioenerg. Biomembr. 16*, 583-595.
Sun, I. L., Navas, P., Crane, F. L., & Morre, D. J., & Low, H. (1987) *J. Biol. Chem. 262*, 15915-15921.
Thorstensen, K. (1988) *J. Biol. Chem. 263*, 16837-16841.
Vuokila, P. T., & Hassinen, I. E. (1988) *Biochem. J. 249*, 339-344.
Vuokila, P. T., & Hassinen, I. E. (1989) *Biochim. Biophys. Acta 974*, 219-222.
Wang, C. S. (1977) *Eur. J. Biochem. 78*, 569-574.
Wang, C. S. (1980) *Biochim. Biophys. Acta 616*, 22-29.
Watkins, J. A., Nunez, M. T., Gaete, V., Alvarez, O., & Glass, J. (1990) *J. Membr. Biol. 119*, 141-149.
Watkins, J. A., Altazan, J. D., Elder, P., Gaete, V., Li, C.-Y., Nunez, M. T., & Glass, J. (1992) *FASEB J. 6*, A190.

Fluorescence-Detected Assembly of the Signal Recognition Particle: Binding of the Two SRP Protein Heterodimers to SRP RNA Is Noncooperative†

Fabiola Janiak,‡ Peter Walter,§ and Arthur E. Johnson*,‡

Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0448

Received December 30, 1991; Revised Manuscript Received April 1, 1992

ABSTRACT: Protein–RNA and protein–protein interactions involved in the assembly of the signal recognition particle (SRP) were examined using fluorescence spectroscopy. Fluorescein was covalently attached to the 3′-terminal ribose of SRP RNA following periodate oxidation, and the resulting SRP RNA-Fl was reconstituted into a fluorescent SRP species that was functional in promoting translocation of secretory proteins across the membrane of the endoplasmic reticulum. Each of the two protein heterodimers purified from SRP elicited a substantial change in fluorescein emission upon association with the modified RNA. The binding of SRP9/14 to singly-labeled SRP RNA-Fl increased fluorescein emission intensity by 41% at pH 7.5 and decreased its anisotropy from 0.18 to 0.16. The binding of SRP68/72 increased the fluorescein anisotropy from 0.18 to 0.23 but did not alter the emission intensity of SRP RNA-Fl. These fluorescence changes did not result from a direct interaction between the dye and protein because the fluorescein remained accessible to both iodide ions and fluorescein-specific antibodies in the complexes. The spectral changes were elicited by specific SRP RNA–protein interactions, since (i) the SRP9/14- and SRP68/72-dependent changes were unique, (ii) an excess of unlabeled SRP RNA, but not of tRNA, blocked the fluorescence changes, and (iii) no emission changes were observed when SRP RNA-Fl was titrated with other RNA-binding proteins. Each heterodimer bound tightly to the RNA, since the K_d values determined spectroscopically and at equilibrium for the SRP9/14 and the SRP68/72 complexes with SRP RNA-Fl were <0.1 and 7 ± 3 nM, respectively. The binding affinity of SRP68/72 for SRP RNA-Fl was unaffected by the presence of SRP9/14, and hence the binding of the heterodimers to SRP RNA is noncooperative in the absence of SRP54 and SRP19. The SRP protein heterodimers therefore associate randomly and independently with SRP RNA to form domains in the particle that are distinct both structurally and functionally. Any cooperativity in SRP assembly would have to be mediated by SRP54 and/or SRP19.

In eukaryotic cells, proteins destined for secretion and those destined to be integral membrane proteins are initially synthesized on cytoplasmic ribosomes. A nascent chain of such a protein is recognized when its signal sequence emerges from the ribosome and is bound to a ribonucleoprotein complex termed the signal recognition particle (SRP)[1] [Walter et al., 1981; for a review, see Walter and Lingappa (1986)]. The association of SRP with the ribosomal complex slows further

† This work was supported by National Institutes of Health Grants GM 26494 (A.E.J.) and GM 32384 (P.W.).

* Address correspondence to this author at the Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, OK 73019.
‡ University of Oklahoma.
§ University of California.

[1] Abbreviations: SRP, signal recognition particle; SRP RNA-Fl, SRP RNA with a fluorescein dye covalently attached to the 3′-terminal ribose; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

0006-2960/92/0431-5830$03.00/0

nascent chain elongation (Walter & Blobel, 1981; Wolin & Walter, 1989), during which time the elongation-arrested complex diffuses to the membrane of the endoplasmic reticulum (ER). There, an interaction between SRP and an integral ER membrane protein termed the SRP receptor or docking protein (Gilmore et al., 1982; Meyer et al., 1982) results in the binding of the ribosome to the ER membrane, the release of both SRP and SRP receptor from the ribosome, the resumption of protein chain elongation, and the initiation of translocation (Gilmore et al., 1982b). The targeting of these ribosomes to the ER membrane also requires GTP (Connolly & Gilmore, 1986, 1989; Hoffman & Gilmore, 1988; Wilson et al., 1988; High et al., 1991a), and the ultimate dissociation of SRP from the SRP receptor requires GTP hydrolysis (Connolly et al, 1991). The sites on the ER membrane at which translocation and integration occur have been termed translocons (Walter & Lingappa, 1986), and photo-cross-linking experiments have shown that nascent chains in the process of either translocation or integration are adjacent to a discrete subset of ER membrane proteins that are presumably components of the putative translocans (Krieg et al., 1989; Wiedmann et al., 1987, 1989; Thrift et al., 1991; High et al., 1991b).

SRP contains 6 different polypeptides and 1 300-nucleotide RNA molecule termed the SRP RNA or 7SL RNA (Walter & Blobel, 1982), and the functions of SRP have been localized to specific components or domains of the SRP. The signal sequence of the nascent chain binds to the 54-kDa protein subunit of SRP (SRP54), as shown by nascent chain cross-linking to SRP54 via photoreactive lysine analogs incorporated into the signal sequence (Krieg et al., 1986; Kurzchalia et al., 1986). Subsequent experiments have shown that the signal sequence binding site is located in the methionine-rich domain of SRP54 (Zopf et al., 1990; High & Dobberstein, 1991). The same domain of SRP54 also contains an RNA-binding site (Zopf et al., 1990; Römisch et al., 1990), but SRP54 association with SRP RNA requires the 19-kDa subunit (SRP19) (Walter & Blobel, 1983a; Siegel & Walter, 1985). The 9- and 14-kDa subunits of SRP are purified as a heterodimer (SRP9/14) (Walter & Blobel, 1983a) and are required for elongation arrest (Siegel & Walter, 1985, 1986). The 68- and 72-kDa subunits of SRP are also purified as a heterodimer (Walter & Blobel, 1983a), and functional SRP68/72 is required for the targeting of the ribosomal complex to the ER membrane (Siegel & Walter, 1985, 1988a). SRP has an elongated rodlike structure with SRP RNA extending throughout the particle (Andrews et al., 1985, 1987), and footprinting experiments have identified the binding sites of the SRP proteins on the SRP RNA (Siegel & Walter, 1988b; Strub et al., 1991). Taken together, the data suggest that the signal sequence binds to SRP54 at one end of an elongated SRP, while the other end of SRP, containing SRP9/14 and the 3′ and 5′ ends of the RNA, interacts with the ribosome to effect elongation arrest.

The assembly of the purified components of SRP into a functional particle can be accomplished in vitro (Walter & Blobel, 1983a; Siegel & Walter, 1985). The much larger bacterial ribosomal subunits have also been reconstituted from their purified components (Nomura et al., 1969; Mizushima & Nomura, 1970; Nowotny & Nierhaus, 1982, 1988), and these studies revealed that the association of the ribosomal proteins with mRNA occurred in a specific order and that the binding of proteins to the complex was often cooperative. Since SRP is also a ribonucleoprotein complex, it is conceivable that SRP assembly may require an ordered sequence of protein–RNA and protein–protein interactions. Consistent with this view, SRP54 will not associate with the SRP RNA in the absence of SRP19 (Walter & Blobel, 1983a; Siegel & Walter, 1985). Moreover, since neither SRP9 or SRP14 will bind by itself to SRP RNA, a specific protein–protein interaction must be required to stabilize the SRP9/14 interaction with RNA (Strub & Walter, 1989, 1990). Similarly, SRP68 will not bind to SRP RNA in the absence of SRP72 (Herz et al., 1990), even though most of SRP72 was released, and the SRP68 fragments remained bound, after limited proteolytic digestion of SRP68/72 bound to SRP RNA (Scoulica et al., 1987). The SRP proteins therefore appear to bind to SRP RNA in pairs. A linkage between the binding of these pairs of proteins was indicated when the addition of excess SRP RNA did not reduce the activity of a sample of SRP (Walter & Blobel, 1983a). This suggests that SRP proteins bind cooperatively, i.e., bind most tightly to an RNA that is already complexed to other SRP proteins.

In order to gain further insight into the interactions that mediate SRP assembly, we have used a spectral probe at the 3′ terminus of SRP RNA to monitor the binding of SRP9/14 and SRP68/72 to SRP RNA. This reporter group allowed us to detect protein-dependent changes in RNA conformation, and to characterize both heterodimer–RNA and heterodimer–heterodimer interactions.

Experimental Procedures

SRP and SRP Proteins. SRP was purified and SRP proteins were isolated as described (Walter & Blobel, 1983; Siegel & Walter, 1985), except that the sucrose density sedimentation was omitted in the purification of SRP. Total protein fractions (1 mL of each) containing all six SRP proteins were obtained following anion-exchange chromatography (Walter & Blobel, 1983a) and, when used directly for reconstitution experiments, were dialyzed at 4 °C into 50 mM Hepes (pH 7.5), 0.5 M KOAc, 5 mM $Mg(OAc)_2$, and 0.01% (w/v) Nikkol (octaethylene glycol mono-*N*-dodecyl ether; Nikko Chemicals Co., Tokyo, Japan). Individual SRP proteins were purified as described by Siegel and Walter (1985), except that the elution buffers contained 10% (v/v) glycerol and lacked dithiothreitol. Fractions containing purified proteins were split into 10-μL aliquots and stored at −75 °C.

Detection of SRP proteins during purification and determination of the protein concentration of individual solutions were done by SDS–PAGE utilizing the Phastsystem (Pharmacia LKB, Piscataway, NJ). After acid precipitation, samples (typically 0.2–0.6 μg per protein) were resuspended and boiled in sample buffer and then electrophoresed (10 mA for 65 AVh at 15 °C) in the presence of 0.55% (w/v) SDS on a 10–15% Phastgel that had been preelectrophoresed for 1 min. Gels were stained with Coomassie, and concentrations were estimated by eye comparison with the protein content of known amounts of purified SRP.

SRP RNA. SRP RNA was purified directly from canine pancreas SRP (Siegel & Walter, 1985) or was transcribed in vitro from a gene for the canine pancreas SRP RNA that had been cloned behind a T7 promoter in a pSP64 vector designated pSP 7SL (Strub et al., 1991). Prior to transcription, the plasmid was linearized with restriction endonuclease *Xbal* (Promega, Madison, WI). Transcription incubations (3.25 mL) contained (Milligan & Uhlenbeck, 1989) each NTP at 4 mM, 24 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 1 mM sperimidine, 0.5 unit/μL placental RNase inhibitor (RNasin; Promega), 20 μg of linearized plasmid DNA, 90 μg (11 units/μg) of T7 RNA polymerase [prepared according to Davanloo et al. (1984) and Grodberg and Dunn

(1988) using BL21/pAR1219 cells kindly supplied by Dr. F. W. Studier], and 50–100 μCi of either [^{3}H]GTP, [^{3}H]ATP, or [^{3}H]UTP (ICN Biomedicals Inc., Costa Mesa, CA, or New England Nuclear, Wilmington, DE). After incubation for 2.5 h at 37 °C, the sample was extracted twice with phenol/chloroform (1:1 v/v) and once with chloroform/isoamyl alcohol (24:1 v/v) and then precipitated in ethanol.

SRP RNA was purified by FPLC at room temperature using a 1-mL Mono Q anion-exchange column (HR 5/5, Pharmacia), a flow rate of 0.15 mL/min, and an 8-mL linear gradient between 0.61 and 0.65 M NaCl in 10 mM $MgCl_2$ and 30 mM Hepes (pH 7.6). Fractions (1.0 mL) containing SRP RNA were pooled, and the RNA was precipitated in ethanol. After centrifugation, the RNA pellets were dried with nitrogen, resuspended in deionized water to an A_{260} of 200–250, and stored at −75 °C. RNA concentrations were determined assuming a molar extinction coefficient at 260 nm of 2×10^6 M^{-1} cm^{-1}. The tritium content ranged from 40 000 to 80 000 cpm per A_{260} unit of RNA.

Fluorescent SRP RNA. SRP RNA was labeled at its 3′-terminal ribose largely as described elsewhere (Odom et al., 1980; Wells & Cantor, 1977). In vitro-transcribed SRP RNA (50–100 μL) was oxidized at a concentration of 200–250 A_{260} units/mL in 0.1 M $NaIO_4$/0.1 M sodium phosphate (pH 7.0) for 90 min at room temperature in the dark. Excess periodate was precipitated with 0.2 M KCl at 0 °C for 10 min, and the precipitate was removed by sedimentation in a microfuge. After dialysis into 50 mM sodium phosphate (pH 7.0) for 4 h at 4 °C, the RNA was reacted with 2 mM 5-[[[2-(carbohydrazino)methyl]thio]acetyl]aminofluorescein (Molecular Probes, Eugene, OR) for 2 h at room temperature in the dark in the presence of 0.5–1.0 mM freshly-made sodium cyanoborohydride. The RNA was then ethanol-precipitated 3–4 times in succession to remove excess unreacted dye, and finally resuspended in 1 mL of 400 mM NaCl, 10 mM $MgCl_2$, and 30 mM Hepes (pH 7.6) for chromatography.

Fluorescein-labeled SRP RNA (termed SRP RNA-Fl) was separated from unmodified RNA using the Mono Q column and the following elution regimen: 0.61–0.63 M NaCl over 4 mL; 0.63 M NaCl maintained for 5 mL; 0.63–0.70 M NaCl over 5 mL; 0.7–1.0 M NaCl over 5 mL. SRP RNA that had been labeled with the fluorescein reagent eluted in two broad peaks centered at 0.67 and 0.76 M NaCl. The RNA in each peak was pooled and precipitated with ethanol. The resulting pellets were resuspended to approximately 20 A_{260} units/mL in 20–50 μL of deionized water and stored at −75 °C. To avoid significant losses due to the small sample volume, these samples were not dialyzed after resuspension.

The specific activities (cpm/A_{260} unit) of the fluorescent RNAs were the same as those of the original unmodified RNAs. To determine the fluorescence emission intensity (in photons detected per second) per A_{260} unit of individual RNA fractions, an aliquot was completely hydrolyzed in 0.1 M KOH for 30 min at 37 °C, and the sample intensity was measured in this solvent.

The stability of the dye–ribose linkage was evaluated by incubating SRP RNA-Fl for 30 min in 0.5 M KOAc, 20 mM Hepes (pH 7.5), 5 mM $Mg(OAc)_2$, and 0.01% (w/v) Nikkol at either 0 °C or room temperature, followed by gel filtration chromatography [Biogel P6 (Bio-Rad Laboratories, Richmond, CA), 0.5 cm i.d. × 16 cm] in 20 mM Hepes (pH 7.6)/5 mM $MgCl_2$ at 4 °C to separate free dye from RNA-bound dye. The fluorescein emission intensity of each fraction (370 μL) was measured, and the extent of hydrolysis of the dye from the RNA was given by (total intensity eluted in the included volume)/(total intensity eluted from the column).

Reconstitutions. SRP RNA and total SRP proteins were reconstituted into SRP as described (Walter & Blobel, 1983a; Siegel & Walter, 1985) with the following changes: SRP proteins (120–150 nM) and SRP RNA (116 nM) were combined in 20 mM Hepes (pH 7.5), 0.5 M KOAc, 5 mM $MgCl_2$, and 0.01% (w/v) Nikkol in a total volume of 6 μL. The samples were incubated for 10 min on ice, followed by 10 min at room temperature instead of 37 °C. The lower temperature was used to minimize loss of the dye from the fluorescent RNA.

Translations. Wheat germ extract and EDTA- and salt-extracted microsomal membranes were prepared as described elsewhere (Erickson & Blobel, 1983; Walter & Blobel, 1983b,c). Messenger RNA coding for preprolactin was synthesized by in vitro transcription as described previously (Krieg et al., 1986, 1989), except that the concentration of GTP was initially 50 μM and was raised to 450 μM after 90 min of incubation. Translation incubations (25 μL, 26 °C) contained 140 mM KOAc, 3 mM $Mg(OAc)_2$, 0.2 mM spermidine, 20 mM Hepes (pH 7.5), 8 μM *S*-adenosylmethionine (Sigma), 0.0024% (w/v) Nikkol, 1 mM glutathione, preprolactin mRNA (0.2 μL of a transcription mix), and 25 μCi of [^{35}S]methionine (Translabel, ICN). Wheat germ extract, amino acids, an energy-generating system, RNase inhibitor, and protease inhibitors were added as described in Erickson and Blobel (1983). Purified SRP, reconstituted SRP, and EDTA- and salt-washed microsomal membranes (2 equiv) were added as indicated. After incubation for 10 min, initiation was inhibited by adding 7-methylguanosine 5′-monophosphate to 4 mM and edeine to 10 μM. Translation was stopped after 30 min by placing the samples on ice. To determine the extent of translocation in a sample, $CaCl_2$ and proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) were added where indicated to 10 mM and to 1 mg/mL, respectively, followed by incubation on ice for 60 min. After precipitation with cold trichloroacetic acid, the samples were washed with acetone/0.1 M HCl (19:1 v/v), redissolved in sample buffer (65 °C, 30 min), and then boiled prior to analysis by SDS–PAGE on a 10–20% gradient gel. Radioactive protein species were detected using fluorography.

Immunoprecipitations. Polyclonal antibodies specific for fluorescein were obtained by immunizing a goat with keyhole limpet hemocyanin that had been reacted with fluorescein isothiocyanate using standard techniques (Hudson & Hay, 1980) and purifying the resultant IgG fraction from the serum using DEAE-cellulose (Hudson & Hay, 1980). Anti-fluorescein antibodies were covalently bound to protein G–Sepharose as described elsewhere (Harlow & Lane, 1988). To determine the extent of covalent attachment of fluorescein to RNA in SRP RNA-Fl samples, 15 μL of the immobilized antibodies was incubated (1 h, 4 °C) with 0.4–0.8 μM SRP RNA-Fl in 20 μL of 20 mM Tris (pH 7.5)/150 mM NaCl. After sedimentation to remove the Sepharose beads, the emission intensity of the supernatant was measured, and its RNA content was determined by measuring the tritium cpm. Under these conditions, about 70–80% of the SRP RNA-Fl was immunoprecipitated.

Fluorescence Spectroscopy. All spectral measurements were made in 4 mm × 4 mm quartz cuvettes using a photon-counting SLM 8000C spectrofluorometer (SLM Instruments, Inc., Urbana, IL). Samples were excited at 490 nm (4- or 8-nm bandpass), and emission was monitored at 520 nm (16-nm bandpass). The sample temperature was maintained at 5 °C, and the sample compartment was flushed with a

steady stream of nitrogen to prevent the condensation of water on the cuvette faces. To avoid photodegradation of the sample, the shutters were kept closed except during measurements.

For steady-state anisotropy measurements, Glan–Thompson prism polarizers were placed into the excitation and emission light paths, and the anisotropy was determined using the L format. The anisotropy, r, was calculated using

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

where the grating factor $G = I_{HV}/I_{HH}$. I_{VH} represents the emission intensity observed with a vertical excitation polarizer and a horizontal emission polarizer; I_{HH}, I_{VV}, and I_{HV} are defined analogously. The intensities of a dye-free, but otherwise equivalent, sample were subtracted from the appropriate intensities in the fluorescein-containing sample to obtain the net fluorescein emission intensities.

The fluorescence lifetime experiments were performed using an ISS Model K2 multifrequency phase fluorometer (ISS, Champaign, IL). The samples were excited with the 488-nm line of a Spectra-Physics Model 2045, 15-W argon ion laser (Spectra-Physics, Piscataway, N.J.). The fluorescence lifetimes were determined by the best fit to a continuous distribution model (Alcala et al., 1987).

Titrations. Titrations of SRP RNA-Fl with SRP proteins were carried out, unless indicated otherwise, using an initial sample volume of 230 μL in 60 mM Hepes (pH 7.5), 0.5 M KOAc, 5 mM $Mg(OAc)_2$, and 0.01% (w/v) Nikkol. Samples were mixed using a Teflon-coated 2 mm × 2 mm magnetic stirrer as described by Dell et al. (1990). The fluorescence intensity or anisotropy of each sample was first determined in the absence of protein and designated F_0 and r_0, respectively. Then, after each addition of titrant, the samples were thoroughly mixed and allowed to reequilibrate at 5 °C before measuring the emission intensity (F) or anisotropy (r) at a particular concentration of added protein. The time necessary for reequilibration was measured routinely and found to be less than 10 min. The signal of a fluorescein-free blank was subtracted from the sample signal at each point. For titrations monitored by the emission intensity, the net intensity was corrected for dilution due to the addition of the titrant. The intensity data shown in the figures were also corrected for dye hydrolysis from the RNA and for adsorption to the cuvette by normalizing sample data at each point with data obtained from a parallel SRP RNA-Fl sample titrated only with buffer. The hydrolysis of the dye from the RNA was minimized by doing the titrations quickly and at 5 °C, and the maximum correction by the buffer-titrated fluorescein-containing sample was less than 5% in all experiments.

Determination of K_d. K_d values were calculated from the protein concentration dependence of the fluorescence-detected binding of protein to SRP RNA-Fl. Each SRP RNA was assumed to bind one SRP9/14 and one SRP68/72. The fraction of SRP RNA-Fl bound to protein at any point in a titration was given by $(F - F_0)/(F_{max} - F_0)$ for SRP9/14·SRP RNA-Fl, and by $(r - r_0)/(r_{max} - r_0)$ for SRP68/72·SRP RNA-Fl since there was no change in fluorescein intensity upon complex formation. F_{max} and r_{max} are the maximum values of intensity and anisotropy observed in the presence of an excess or protein. For the SRP68/72 titrations, the dissociation constant was then calculated using a nonlinear regression analysis program, ENZFITTER (Imperial College of Science and Technology, London, UK), to fit the data to the Scatchard equation. The reported K_d values are maximum values because all SRP RNA-Fl molecules, as well as all protein molecules, were assumed to be active and to participate in complex formation.

Fluorescence Quenching. Collisions between fluorescent chromophores and certain solutes, such as iodide ions, result in a quenching of fluorescence [e.g., see Lehrer and Leavis (1978), Adkins et al. (1983), and Isaacs et al. (1986)]. Thus, the exposure of a particular fluorophore to the solvent and dissolved solute can be examined by measuring the emission intensity as a function of quencher concentration. For steady-state collisional quenching of dyes located in equivalent environments, a linear plot is obtained when data are analyzed according to the Stern–Volmer law:

$$F_0/F = K_{SV}[Q] + 1 \quad (2)$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of quencher at concentration [Q], and K_{SV} is the Stern–Volmer quenching constant.

The extent of iodide ion quenching of fluorescein was determined by monitoring the emission intensity of a sample as it was titrated with 2 M KI in 50 mM Hepes (pH 7.5), 0.5 M KOAc, 5 mM $Mg(OAc)_2$, and 0.1 mM Na_2SO_3 up to a final concentration of less than 0.1 M KI. The effects of ionic strength on dye emission were quantified by titrating a parallel sample with 2 M KCl instead of 2 M KI. After subtraction of the intensities of the appropriate fluorescein-free samples, the emission intensities were corrected for dilution due to addition of the KI or KCl solutions. The iodide ion-dependent quenching of fluorescein was then given by the difference between the net dilution-corrected emission intensities of the samples titrated with equal amounts of KI and KCl.

Materials. Unfractionated *Escherichia coli* transfer RNA (Boehringer) was used without further purification. Pure samples of elongation factor Tu from *E. coli* and of elongation factor 1α from rabbit reticulocyte were kindly provided by Dr. D. L. Miller and Dr. W. C. Merrick, respectively.

Results

Preparation and Purification of in Vitro-Transcribed SRP RNA. To obtain large amounts for modification, SRP RNA was transcribed from plasmid DNA in vitro. Purification of this material on an anion-exchange column at a slow flow rate yielded three peaks of SRP RNA, which suggests that the transcription incubation yielded different RNA species (Figure 1A). These chromatographically-separable RNA species differed only in their conformations, because RNA from different peak fractions migrated as single bands with the same mobilities on a 6% polyacrylamide gel in the presence of 7 M urea (data not shown). Furthermore, each of the RNA species could be reconstituted into SRP particles that promoted translocation of preprolactin across ER membranes, though the translocation activity of reconstituted SRP was reduced by 70% when SRP RNA from fractions 30–36 was used instead of SRP RNA from fractions 28–29 (data not shown). Different conformational states of SRP RNA have also been observed when separations have been done using electrophoresis rather than chromatography (Zwieb & Ullu, 1986).

SRP RNA that had been extracted from purified SRP eluted from this resin in a single peak at 0.63 M NaCl (data not shown). Thus, only RNA that eluted from the resin at a NaCl concentration less than 0.64 M was used in the fluorescent-labeling reactions.

Fluorescent SRP RNA. Purified in vitro-transcribed SRP RNA was oxidized at its 3′-terminal ribose using periodate and then reacted with a hydrazine-containing fluorescein derivative. The fluorescent SRP RNA, designated SRP RNA-Fl, was separated from nonfluorescent RNA by ion-exchange chromatography. The fluorescein-labeled SRP RNA

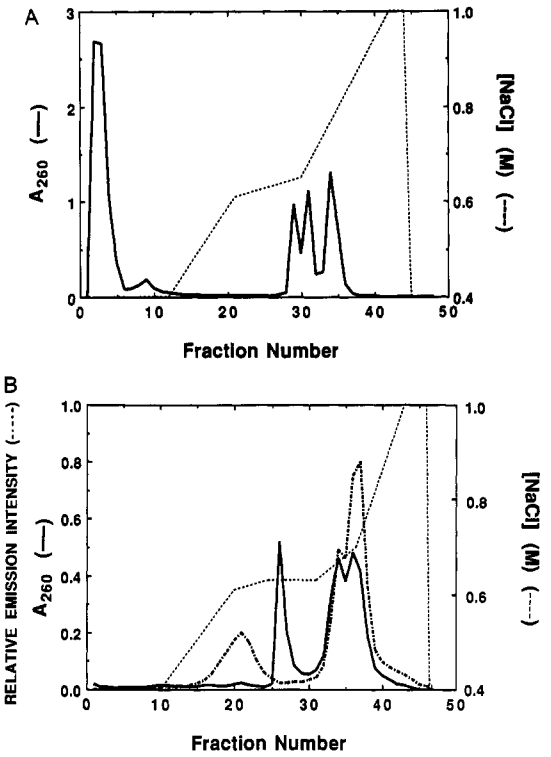

FIGURE 1: (A) Purification of in vitro-transcribed SRP RNA. SRP RNA was transcribed as described under Experimental Procedures and purified by ion-exchange chromatography over a Mono Q resin. The salt gradient is shown, and the fraction size was 1.0 mL. The peak eluting at 0.4 M NaCl contained unincorporated NTPs. The peak fractions containing SRP RNA were pooled separately (fractions 28–29, 30–32, and 33–36). (B) Purification of fluorescent-labeled SRP RNA. SRP RNA from fractions 28–29 of (A) was labeled and then purified as described under Experimental Procedures. The salt gradient is shown, and the fraction size was 1.0 mL. The three RNA peak fractions in this separation were identified by the absorbance at 260 nm and pooled separately as peak A (fractions 25–28), peak B (fractions 32–35), and peak C (fractions 36–39).

eluted in two separate peaks (0.67–0.76 M NaCl; peaks B and C, Figure 1B) that were distinct from the elution position of unmodified SRP RNA (0.63 M NaCl; peak A, Figure 1B) and of unreacted fluorescein reagent (fractions 17–23 in Figure 1B). The ratio of fluorescence intensity to A_{260} (or radioactivity) of the peak C fluorescent RNA was approximately twice as high (1.8–2.2-fold for different RNA preparations) as that of the peak B fluorescent RNA, which indicates that the SRP RNA-Fl in peak C has twice as many dyes per RNA as the RNA in peak B. Since oxidation of the RNA with sodium periodate creates a dialdehyde structure at the 3′-terminal ribose with two reactive functional groups, we conclude that the peak C RNA has reacted covalently with two fluorescein reagent molecules. Both the singly-labeled and doubly-labeled fluorescent SRP RNA species exhibited fluorescence changes upon binding to the SRP heterodimers, although the magnitudes of the spectral changes differed for these RNAs (see below). Both fluorescent RNA species, or a mixture of the two, could therefore be used to monitor complex formation spectroscopically.

To determine if all RNA molecules in a SRP RNA-Fl sample contained fluorescein, SRP RNA-Fl was immunoprecipitated using anti-fluorescein antibodies that were covalently linked to protein G–Sepharose. Under the conditions of this experiment, 70–80% of the SRP RNA-Fl bound to the resin. After removal of the resin, the fluorescence and radioactivity remaining in the supernatant were determined. The ratio of fluorescence intensity to radioactivity in a sample of purified SRP RNA-Fl from either peak B or peak C of Figure 1B was the same before and after immunoprecipitation (data not shown), which indicates that fluorescein is attached covalently to *each* RNA molecule in a purified SRP RNA-Fl sample.

Stability of SRP RNA-Fl. The stability of the dye–ribose bond was investigated by incubating SRP RNA-Fl at different temperatures and times, followed by separating the free dye from the RNA-bound dye by gel filtration chromatography. Less than 1% of the dye eluted in the included volume when SRP RNA-Fl was kept at 0 °C for 30 min, and less than 10% of the dye eluted in the included volume after incubation at room temperature for 30 min (data not shown). When SRP RNA-Fl was incubated at 37 °C for 10 or 60 min, the amount of dye hydrolyzed from the RNA was 8% and 27%, respectively (data not shown). Our reconstitution incubations were therefore done at room temperature for 10 min, and titrations were done at 5 °C, so that the amount of dye hydrolyzed in our experiments was negligible (<5%).

Fluorescent SRP Is Active. To determine whether the modified SRP RNA would still interact with SRP proteins to form functionally active SRP complexes, aliquots from each of the two SRP RNA-Fl species (peaks B and C, Figure 1B) were reconstituted with total SRP proteins and, without further purification, were added to a wheat germ translation assay in the presence of both mRNA coding for the secretory protein preprolactin and EDTA- and salt-washed microsomal membranes. Since the signal peptide is cleaved from preprolactin during translocation, prolactin polypeptides should be found only in samples that contain functional SRP. Furthermore, prior to analysis by SDS–PAGE, all samples were subjected to extensive digestion with proteinase K so that only the polypeptides that had been translocated across the ER membrane into the microsomal vesicles would be protected from digestion.

As is evidenced by the presence of prolactin in lanes 3–5 of Figure 2, both RNA species were reconstituted into particles that were active in targeting and translocating preprolactin. Hence, each fluorescent-labeled RNA must interact functionally with the SRP proteins. However, the translocation activity of the fluorescent SRP (the extent of prolactin formation was estimated by densitometry of autoradiograms such as that shown in Figure 2) was only about 25% that of SRP reconstituted with unmodified SRP RNA that had been exposed to the same reaction and purification conditions as SRP RNA-Fl. The presence of the dye therefore reduces the assembly or the specific activity of SRP. The cause of the decrease in activity has yet to be determined.

Fluorescence-Detected Association of SRP9/14 with SRP RNA-Fl. Since the binding site for SRP9/14 is located close to the 3′ end of the RNA (Strub et al., 1991), SRP RNA-Fl was titrated with the heterodimer to determine if complex formation could be detected by a change in the fluorescence signal of the dye attached to the 3′-terminal ribose. As shown in Figure 3, the binding of SRP9/14 to SRP RNA-Fl caused a large increase in fluorescein emission intensity that was saturable. The emission intensity increase averaged 41% for singly-labeled SRP RNA-Fl (peak B, Figure 1B) and 11% for doubly-labeled SRP RNA-Fl (peak C, Figure 1B) at pH 7.5 (Table I). For samples that contained a mixture of the two fluorescent species, the SRP9/14-dependent intensity increase was between 11 and 41%. The magnitude of the protein-dependent intensity change was also pH-dependent, since the SRP9/14-dependent intensity increase for one SRP RNA-Fl sample averaged 42% at pH 7.0 and 21% at pH 7.5. The wavelength of the emission intensity maximum was not altered

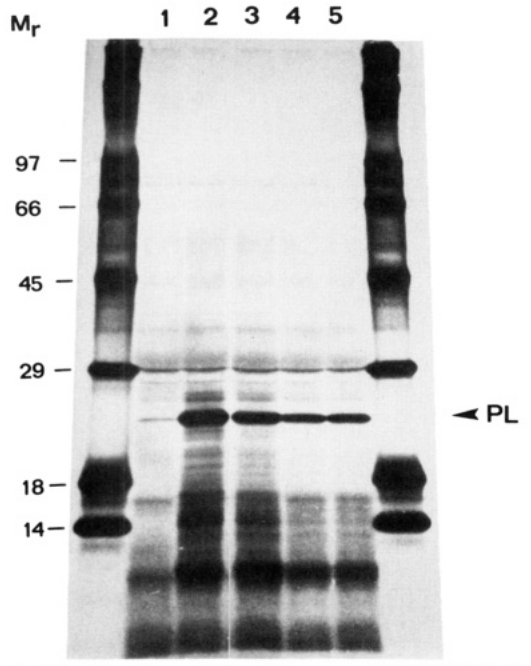

FIGURE 2: Translocation activity of fluorescent-labeled SRP. In vitro translation assays (25 μL) contained preprolactin mRNA, wheat germ extract, [^{35}S]Met, and EDTA- and salt-washed microsomal membranes. SRP was added as indicated below. Samples were translated, digested with proteinase K, and analyzed by SDS–PAGE as described under Experimental Procedures. The radioactive bands were made visible by fluorography. Lane 1, no SRP added; lane 2, 25 nM SRP purified from canine pancreas; lane 3, unlabeled SRP reconstituted from total SRP proteins and 28 nM SRP RNA from peak A (Figure 1B); lane 4, fluorescent-labeled SRP reconstituted from total SRP proteins and 28 nM SRP RNA-Fl from peak B (Figure 1B); lane 5, fluorescent-labeled SRP reconstituted from total SRP proteins and 28 nM SRP RNA-Fl from peak C (Figure 1B). The following ^{14}C-labeled proteins were used as molecular mass markers: phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18 kDa; lysozyme, 14 kDa.

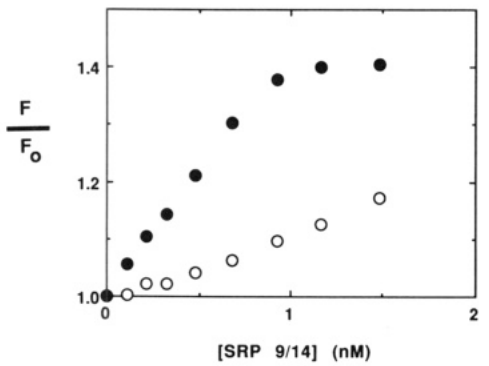

FIGURE 3: Titration of SRP RNA-Fl with SRP9/14. Singly-labeled SRP RNA-Fl (initially 1.2 nM) in the absence (●) or presence (○) of a 10-fold molar excess of unlabeled SRP RNA was titrated with SRP9/14 as described under Experimental Procedures. The data shown are from two titrations done in parallel.

when SRP9/14 bound to SRP RNA-Fl. The change in intensity was also accompanied by a decrease in anisotropy when SRP9/14 associated with SRP RNA-Fl. For singly-labeled RNA at pH 7.5, the average anisotropy decreased from 0.177 to 0.155 upon binding SRP9/14, while the corresponding change for doubly-labeled RNA was from 0.169 to 0.149 (Table I). The formation of the SRP9/14·SRP RNA-Fl complex therefore alters the emission, and hence the environment, of a fluorescein dye at the 3′ end of the RNA.

The SRP9/14-dependent emission changes reached their maximum levels when the calculated molar ratio of SRP9/14 to SRP RNA-Fl in a sample was between 1:1 and 3:1. Since there was uncertainty associated with the measurement of the SRP9/14 concentration and there is no measure of protein activity, this range is reasonably consistent with the expected formation of SRP particles that have an equimolar composition of components.

Table I: Summary of SRP RNA-Fl Fluorescence Emission in the Presence and Absence of SRP Protein Heterodimers[a]

	none	SRP9/14	SRP68/72
emission intensity[b]	1.00[c]	1.41[c]	1.00[c]
	1.00[d]	1.11[d]	1.00[d]
anisotropy	0.177[c]	0.155[c]	0.234[c]
	0.169[d]	0.149[d]	0.226[d]
K_{sv} (M^{-1})	3.9	6.3	3.8
τ (ns)	3.9	3.9	3.9

[a] Data were obtained at 5 °C in 0.5 M KOAc, 60 mM Hepes (pH 7.5), 5 mM $Mg(OAc)_2$, and 0.01% (w/v) Nikkol. Standard errors (n = 3–11 separate experiments) for intensity measurements were less than ±0.03; standard errors for anisotropy data were ±0.009 for samples containing SRP68/72, ±0.011 for doubly-labeled SRP RNA-Fl preparations, and ±0.005 for the others; standard errors in K_{sv} values were ±0.1 for SRP68/72 samples and ±0.03 for the others. The uncertainty in the lifetime measurements was ±0.2 ns. [b] Relative to the emission intensity of the SRP RNA-Fl species in the absence of SRP proteins. [c] Data obtained using a sample of SRP RNA-Fl that had only a single fluorescein dye attached to the 3′-terminal ribose. [d] Data obtained using a sample of SRP RNA-Fl that had two fluorescein dyes attached to the 3′-terminal ribose. The lifetime and quenching data were also obtained using this RNA.

The large SRP9/14-dependent increase in emission intensity was not associated with an increase in the lifetime of the dye, for the fluorescein lifetime of SRP RNA-Fl was found to be 3.9 ns both in the presence and in the absence of SRP9/14 (Table I). Thus, the fluorescence intensity change results from an increase in the absorptivity of the dye rather than an increase in its quantum yield. This indicates that the binding of SRP9/14 to SRP RNA-Fl alters the local pH near the fluorescein and/or the pK_a of the monoanion form of fluorescein, since the absorptivity of fluorescein is dictated by the extent of its ionization (Mercola et al., 1972). (This change in fluorescein environment is caused by a change in RNA conformation that is effected allosterically by SRP9/14, as shown below.) This type of spectral change also occurs when a fluorescein-labeled aminoacyl-tRNA associates with elongation factor Tu·GTP (Adkins et al., 1983; Hazlett et al., 1989; Janiak et al., 1990).

Specificity of Fluorescence Changes. The fluorescence intensity increase of 41% shown in Figure 3 resulted from a specific protein–RNA interaction because it was observed only with SRP RNA-Fl and SRP9/14. When SRP RNA-Fl was titrated with SRP68/72, SRP19, or SRP54, the intensity increase was less than 8% (data not shown). Furthermore, no change in emission intensity or steady-state anisotropy was observed when SRP RNA-Fl was titrated in the presence of GTP with either the prokaryotic elongation factor Tu or the eukaryotic factor 1α (data not shown), both of which are known to bind to aminoacyl-tRNAs and to induce conformational changes in the tRNAs (Adkins et al., 1983; Johnson et al., 1986; Dell, 1987; Janiak et al., 1990).

The specificity of the SRP9/14 interaction for SRP RNA was also indicated by the fact that a 50-fold molar excess of tRNA did not alter the magnitude of the fluorescence change or its dependence upon SRP9/14 concentration (data not shown). Thus, the tRNA did not compete with SRP RNA-Fl for binding to SRP9/14. In contrast, a 10-fold excess of nonfluorescent SRP RNA in a sample of SRP RNA-Fl greatly reduced the SRP9/14-dependent intensity increase (Figure

3). The extent of the SRP RNA-dependent reduction in emission intensity (80.4–95.6% when SRP9/14 was limiting in the experiment depicted in Figure 3) was consistent with an approximately equal affinity of the SRP9/14 for SRP RNA and SRP RNA-Fl, which indicates that the fluorescein does not significantly influence the strength of the SRP9/14 interaction with SRP RNA.

Affinity of SRP9/14 for SRP RNA-Fl. The binding of SRP9/14 to SRP RNA-Fl was very tight, with a dissociation constant of less than 0.1 nM estimated from the titration data. The K_d could not be measured directly using the fluorescence approach because even at the lowest concentration of SRP RNA-Fl that could be employed (0.1 nM) essentially all added SRP9/14 was bound to RNA.

The high affinity of this interaction was also shown by the slow reversibility of the spectral change. When a 10-fold molar excess of unmodified SRP RNA was added to a sample containing SRP9/14·SRP RNA-Fl, no reduction in emission intensity was observed after 45 min at 5 °C (data not shown). There are two possible explanations for this observation: either SRP9/14 induced an irreversible (or slowly reversible) conformational change in SRP RNA-Fl, or SRP9/14 did not significantly dissociate from the SRP RNA-Fl during the experiment. The latter possibility is supported by two observations. First, the amount of SRP9/14 that was co-immunoprecipitated with SRP RNA-Fl using anti-fluorescein antibodies and detected by silver staining was the same before and 60 min after the addition of a 10-fold molar excess of unlabeled SRP RNA to SRP proteins·SRP RNA-Fl (data not shown). Second, when SRP9/14·SRP RNA was added to an approximately equimolar amount of SRP RNA-Fl, no increase in the emission intensity was observed after 45 min (data not shown). This indicates that little SRP9/14 dissociated from the nonfluorescent RNA and subsequently bound to the fluorescent RNA during this time period. Thus, the dissociation of the SRP9/14·SRP RNA complex is very slow, which is consistent with a high-affinity interaction between SRP9/14 and SRP RNA.

Accessibility of the Fluorescein Dye in SRP9/14·SRP RNA-Fl. The spectral changes caused by the binding of SRP9/14 to SRP RNA-Fl could result either from a direct interaction in which the dye is covered or contacted by the heterodimer or from a conformational change(s) at the 3′ end that is mediated allosterically by SRP9/14 binding elsewhere on the RNA. The first possibility can be examined directly by determining whether the binding of SRP9/14 to SRP RNA-Fl reduced or blocked the probe's exposure to the solvent, specifically its sensitivity to quenching by iodide ions. This approach has been used previously to investigate other protein–nucleic acid complexes [e.g., see Adkins et al. (1983) and Dell (1987)].

SRP RNA-Fl, either in the presence or in the absence of SRP9/14, was titrated with iodide ions, and iodide ion quenching was observed for both samples (Figure 4). The extent of quenching was directly proportional to the concentration of iodide ions in each sample, as is expected for the collisional quenching of a homogeneous sample. After correction for the small ionic strength-dependent changes in emission intensity that were determined in a parallel titration with chloride ions (Figure 4), the Stern–Volmer constant (K_{SV}) for SRP RNA-Fl was 3.9 M^{-1}, while that for SRP9/14·SRP RNA-Fl was 6.3 M^{-1} (Table I). $K_{SV} = k_q\tau$, where τ is the fluorescence lifetime of the dye and k_q is the bimolecular collisional rate constant (Lehrer & Leavis, 1978). Since the lifetime of the fluorescein is the same for SRP RNA-Fl and

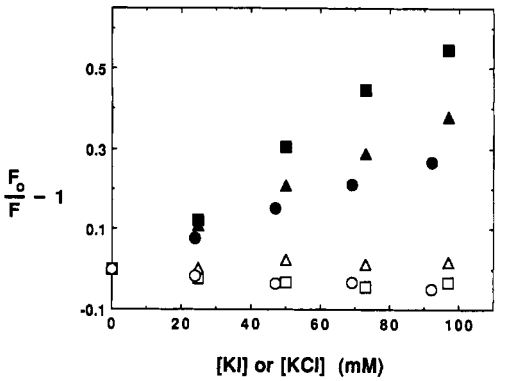

FIGURE 4: Solvent accessibility of the fluorescein in SRP RNA-Fl, SRP9/14·SRP RNA-Fl, and SRP68/72·SRP RNA-Fl. Doubly-labeled SRP RNA-Fl (6.1 nM) was first titrated either with SRP9/14 (to 13 nM; □, ■), with SRP68/72 (to 10 nM; ▲, △), or with buffer (○, ●). The samples were then titrated with a solution containing either 2 M KI (closed symbols) or 2 M KCl (open symbols). The Stern–Volmer constants were obtained from the difference between the KI and KCl titration data as described under Experimental Procedures and are given in Table I.

SRP9/14·SRP RNA-Fl (Table I), k_q is greater for SRP9/14·SRP RNA-Fl than for SRP RNA-Fl. Thus, the binding of the SRP9/14 to the RNA *increases* the frequency of collisions between the probe at the 3′ end of the RNA and iodide ions dissolved in the aqueous solvent.

This observation can be explained in one of two ways. Either the heterodimer alters the conformation of the RNA so that the probe is more exposed to the solvent in the complex than in the naked SRP RNA and/or the binding of the heterodimer to the RNA reduces the negative charge density near the probe so that the electrostatic repulsion of the iodide ions is reduced and the frequency of their collisions with the probe is increased. Since SRP9/14 increases the emission intensity of the fluorescein by increasing its absorptivity (see above), apparently by decreasing the local negative charge density (Mercola et al., 1972), it seems likely that an SRP9/14-dependent neutralization of RNA phosphate charges is at least partially responsible for the higher iodide ion quenching rate in the presence of SRP9/14 than in its absence. The binding of SRP9/14 to the RNA also alters the RNA conformation near the fluorescein because the reduction in anisotropy indicates that the rotational freedom of the dye has increased by a small amount after SRP9/14 binds. Thus, both the SRP9/14-dependent decrease in anisotropy and the SRP9/14-dependent increase in k_q demonstrate that a dye attached to the 3′-terminal ribose does not contact SRP9/14 when the protein binds to the RNA. The SRF9/14-dependent increase in emission intensity therefore results from a change in the conformation at the 3′ end of the RNA that is mediated allosterically by the binding of SRP9/14 to SRP RNA.

The accessibility of the fluorescein dye in SRP RNA-Fl is also shown by the fact that fluorescein-specific antibodies immunoprecipitated SRP RNA-Fl in the presence of SRP9/14 and SRP9/14 was recovered in the immunoprecipitate (data not shown). The binding of the antibodies to the fluorescein dyes could also be followed spectroscopically, since fluorescein emission intensity is quenched by 70% in the dye·antibody complex both in the presence and in the absence of SRP9/14 (data not shown).

Fluorescence-Detected Association of SRP68/72 with SRP RNA-Fl. When pure SRP68/72 was titrated into a solution of SRP RNA-Fl, a large increase in the fluorescein anisotropy of SRP RNA-Fl was observed (Figure 5, Table I). The association of SRP68/72 with SRP RNA-Fl therefore reduced

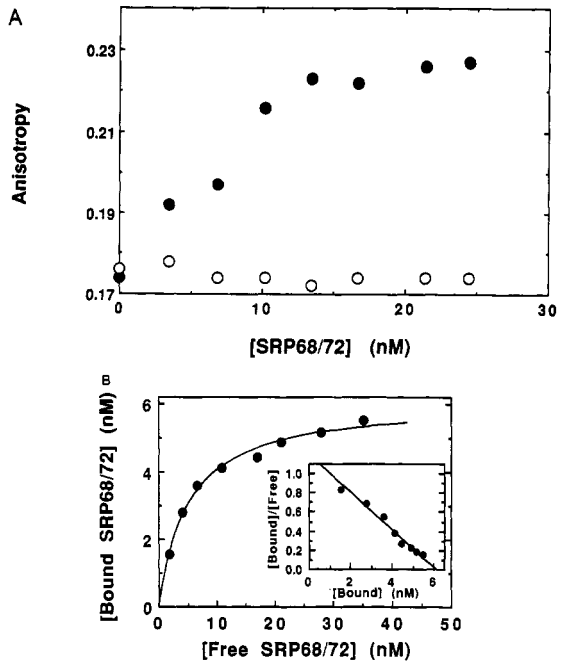

FIGURE 5: Titration of SRP RNA-Fl with SRP68/72. (A) Doubly-labeled SRP RNA-Fl (6.1 nM initially) was titrated with either SRP68/72 (●) or buffer (○) as described under Experimental Procedures. The best-fit SRP68/72·SRP RNA-Fl K_d for this experiment was 5.8 ± 2.1 nM. (B) The data from another titration of doubly-labeled SRP RNA-Fl (6.1 nM) with SRP68/72 (●) are expressed as [bound SRP68/72] ([SRP68/72·SRP RNA-Fl]) versus [free SRP68/72]. The concentration of bound SRP68/72 was determined from the magnitude of the anisotropy change assuming a binding stoichiometry of 1:1, as described under Experimental Procedures. The curve was drawn using the best-fit K_d of 5.1 ± 0.5 nM obtained from the nonlinear least-squares regression analysis of these data. The inset shows these data in a modified Scatchard plot.

the rotational rate of the probe at the 3′ end of the RNA, either by restricting the local freedom of rotation of the dye or by reducing the global rotational rate of the particle, or both.

In contrast, no SRP68/72-dependent change in the fluorescein emission intensity or lifetime of SRP RNA-Fl was observed (Table I). A small intensity increase was observed with some preparations of SRP68/72, but this proved to be due to a contamination of SRP9/14 in those samples of SRP68/72.

Affinity of SRP68/72 for SRP RNA-Fl. Since the binding of SRP68/72 to SRP RNA-Fl elicits a substantial increase in fluorescein anisotropy, the extent of SRP68/72·SRP RNA-Fl complex formation in a sample can be measured spectroscopically. From the concentration dependence of complex formation, the affinity of the heterodimer for the RNA can be determined directly and at equilibrium. The anisotropy of SRP RNA-Fl was therefore monitored as a function of SRP68/72 concentration. Titration data such as those shown in Figure 5 were used to calculate an average K_d value of 7 ± 3 nM for SRP68/72·SRP RNA-Fl. It is clear from these data that SRP68/72 binds more weakly to SRP RNA-Fl than does SRP9/14.

The reversibility of the anisotropy change was also investigated. When a 10-fold molar excess of unmodified SRP RNA was added to a sample containing SRP68/72·SRP RNA-Fl, the anisotropy decreased within 10 min to a level that would be expected if SRP68/72 bound equally well to the two RNA species (Figure 6). The exchange of SRP68/72 between RNAs was shown independently by adding fluorescein-specific antibodies to a sample of SRP68/72·SRP RNA-Fl before and after the addition of a 10-fold molar excess

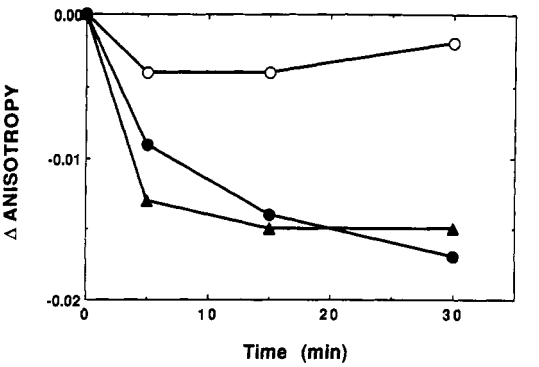

FIGURE 6: Reversibility of SRP68/72 binding to SRP RNA-Fl and SRP9/14·SRP RNA-Fl. Doubly-labeled SRP RNA-Fl (6.1 nM) was incubated with a substoichiometric amount of SRP68/72 (4.8 nM) and, where indicated, an excess of SRP9/14 (7 nM) at 5 °C in 0.5 M KOAc, 60 mM Hepes (pH 7.5), 5 mM $Mg(OAc)_2$, and 0.01% (w/v) Nikkol for 10 min to form SRP68/72·SRP RNA-Fl (○, ●) and SRP68/72·SRP RNA-Fl·SRP9/14 (▲). At t = 0, either buffer (○) or a 10-fold molar excess of unmodified SRP RNA (● and ▲) was added to the samples, and the anisotropy was measured at the time points indicated. The initial anisotropies of the free SRP RNA-Fl, SRP68/72·SRP RNA-Fl·SRP9/4, and SRP68/72·SRP RNA-Fl were 0.175, 0.190, and 0.200, respectively, in this experiment, and were dictated by the limited amount of SRP68/72 in the samples (see Figure 5).

of SRP RNA to the sample. The amount of SRP68/72 in the immunoprecipitate was reduced by more than 50% within 10 min after the addition of the unlabeled RNA (data not shown). These results indicate both that the binding of SRP68/72 to SRP RNA is exchangeable and that the fluorescein has a small, if any, effect on the affinity of SRP68/72 for SRP RNA.

Solvent Accessibility of the Fluorescein in SRP68/72·SRP RNA-Fl. When SRP68/72·SRP RNA-Fl was titrated with iodide ions, collisional quenching of the fluorescein emission was observed with a K_{SV} of 3.8 M^{-1}, essentially the same value found for the quenching of free SRP RNA-Fl (Figure 4; Table I). Thus, the binding of SRP68/72 to SRP RNA-Fl did not alter the rate of iodide ion collision with the fluorescein at the 3′ terminus of the RNA. In addition, when fluorescein-specific antibodies were added to a sample containing SRP68/72·SRP RNA-Fl, SRP68/72 was immunoprecipitated (data not shown). These data indicate that the SRP68/72 does not contact or cover the dye in the SRP68/72·SRP RNA-Fl complex and that the association of SRP68/72 with the RNA does not alter, directly or indirectly, the accessibility of the dye to the solvent.

Cooperativity in the Binding of SRP Heterodimers to SRP RNA. To determine if the prior binding of one heterodimer to SRP RNA influences the binding of the second, the proteins were added to SRP RNA-Fl in each of the two possible sequences. The binding of SRP9/14 caused a decrease in the anisotropy of SRP RNA-Fl, whereas the binding of SRP68/72 caused a large increase (Table I). When SRP RNA-Fl was titrated first with SRP9/14 and then with SRP68/72, or vice versa, the resulting particles were, at least spectroscopically, the same when formed in either order (Figure 7). Also, the SRP9/14-dependent increase in emission intensity was unaffected by the presence or absence of SRP68/72 (data not shown). This suggests that the assembly of SRP heterodimers on SRP RNA occurs randomly, with no required order of addition.

If the binding of SRP heterodimers to SRP RNA-Fl is cooperative, one would expect that the binding of one heterodimer to the SRP RNA would increase the affinity of the particle for the second heterodimer. To determine whether

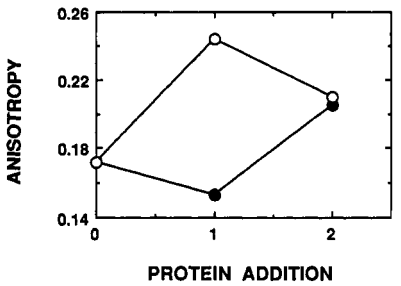

FIGURE 7: Sequential binding of SRP protein heterodimers to SRP RNA-Fl. SRP protein heterodimers were added in two different sequences to samples containing singly-labeled SRP RNA-Fl (initial concentration, 6.1 nM). The anisotropy was measured after each addition. Additions to the (○) sample: SRP68/72 (to 19 nM) at 1, SRP9/14 (to 10 nM) at 2. Additions to the (●) sample: SRP9/14 (to 10 nM) at 1, SRP68/72 (to 19 nM) at 2.

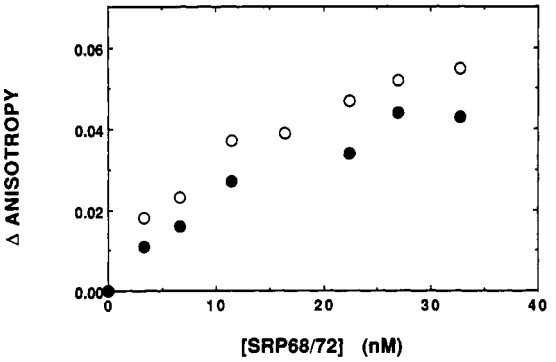

FIGURE 8: SRP68/72 binding to SRP RNA-Fl and SRP9/14·SRP RNA-Fl. Doubly-labeled SRP RNA-Fl (6.1 nM) was incubated with 8 nM SRP9/14 (●) or with buffer (○) for 10 min at 5 °C. The samples were then titrated with SRP68/72 as described under Experimental Procedures, and the change in fluorescein anisotropy was monitored as a function of SRP68/72 concentration.

this occurred, SRP68/72 was added to samples containing either free SRP RNA-Fl or SRP9/14·SRP RNA-Fl. The SRP68/72 concentration dependence of the anisotropy change was similar in both samples (Figure 8), and hence the affinity of SRP68/72 for SRP RNA is the same or slightly weaker in the presence of SRP9/14.

This conclusion is supported by the reversibility data shown in Figure 6. When a 10-fold molar excess of SRP RNA was added to parallel samples of SRP68/72·SRP RNA-Fl and SRP68/72·SRP RNA-Fl·SRP9/14, the anisotropy decreased at the same or a slightly faster rate in the presence of SRP9/14. Although SRP9/14 forms a very stable complex with SRP RNA, the formation of the SRP9/14·SRP RNA complex does not appear to prevent bound SRP68/72 from dissociating from the RNA. Thus, no positively cooperative interactions between SRP68/72 and SRP9/14 are apparent during their binding to SRP RNA-Fl in the absence of SRP19 and SRP54.

Discussion

The assembly of multicomponent ribonucleoprotein particles requires a number of specific, and possibly ordered, protein–protein and protein–RNA interactions in order to obtain a complete, functional particle. An understanding of these interactions not only provides a recipe for particle formation but also provides clues about the molecular mechanisms involved in particle function. If, for example, the binding of a protein to the particle requires a second protein, then the two proteins are structurally coupled in the particle, and this direct or allosteric linkage between two different sites on the particle may be important and utilized during function. Thus, one of the goals of this study was to determine whether SRP assembly, and perhaps function, involves independent domains or conformationally-linked domains within the particle.

Our approach to investigating SRP assembly utilized a fluorescent dye as a spectroscopic probe of interactions between SRP components. This allows us to determine the affinities of SRP components for each other under equilibrium conditions [cf. Abrahamson et al. (1985) and Janiak et al. (1990)] and hence to evaluate directly whether a particular bimolecular association was sensitive to the presence of other components. Furthermore, by covalently attaching the probe at a single site, the 3′ terminus of the SRP RNA, it was possible to identify which SRP proteins could alter the spectral properties, and hence environment, of the dye at that location. The SRP RNA was an obvious choice for positioning a probe because the single RNA molecule extends throughout the SRP (Andrews et al., 1987) and is therefore the most likely candidate for transmitting information from one end of the particle to the other via changes in conformation.

It is important to emphasize that this new approach to examining the assembly, structure, and function of SRP was possible only because the modification of the SRP RNA at its 3′ end did not destroy its activity. After purification of the fluorescent-labeled SRP RNA away from unmodified RNA, the resulting SRP RNA-Fl was successfully reconstituted into SRP that was active in promoting the translocation of preprolactin across the ER membrane (Figure 2). The fluorescent-labeled SRP therefore functioned in both the selection and targeting of nascent secretory proteins for transport, as well as in the interactions with ER membrane components to initiate translocation. On the other hand, the translocation activity of SRP reconstituted with fluorescent-labeled SRP RNA was less than that of SRP reconstituted with unmodified, sham-reacted SRP RNA. It therefore appears that the presence of the fluorescein does interfere to some extent with SRP function, for reasons yet unknown.

Upon binding to SRP RNA-Fl, SRP9/14 and SRP68/72 each altered the fluorescence emission of fluorescein covalently attached to the 3′-terminal ribose (Figures 3 and 5; Table I). The spectral changes elicited by these two heterodimers were different, and hence each change was associated with the formation of a specific SRP RNA–protein complex whose appearance could be monitored by its characteristic fluorescein emission. Consistent with the specificity of these interactions and spectral changes, the presence of excess tRNA or elongation factors did not affect the fluorescence of SRP RNA-Fl or its complexes with the SRP heterodimers.

SRP9/14 association with SRP RNA-Fl resulted in both a large increase in emission intensity and a small decrease in anisotropy (Figures 3 and 7). Even though the binding site for SRP9/14 is located near the 3′ end of the RNA (Strub et al., 1991), these fluorescence changes did not result from SRP9/14 covering or contacting the fluorescein directly because such an interaction would be expected to shield the fluorescein from quenchers and to increase the anisotropy of the fluorescein. Instead, the fluorescein dyes in the SRP9/14·SRP RNA-Fl complex were accessible both to iodide ions and to anti-fluorescein antibodies, and complex formation *decreased* the anisotropy of the fluorescein at the 3′ terminus of the RNA. Thus, the binding of SRP9/14 to SRP RNA allosterically alters the conformation of the RNA at its 3′ terminus. This fluorescence-detected change in RNA conformation is consistent with the observations of Andreazolli and Gerbi (1991), who concluded, on the basis of chemical modification studies, that the conformation of SRP RNA

differs in the presence and absence of SRP proteins.

The footprint-identified binding site of SRP68/72 on the RNA is located about 45 nucleotides away from the 3′ end (Siegel & Walter, 1988b), and the fluorescein accessibility to iodide ions or to anti-fluorescein antibodies was unchanged when SRP68/72 bound to SRP RNA-Fl (Table I). This indicates that SRP68/72 does not cover or contact the fluorescein directly. Since the anisotropy of the dye increased substantially upon SRP68/72·SRP RNA-Fl complex formation, the binding of SRP68/72 to SRP RNA-Fl decreased either the overall rotational rate of the SRP RNA-Fl and/or the local rotational rate of the dye through a long-range allosteric interaction between the 3′ end of the RNA and its binding site for SRP68/72. Our current data do not distinguish between these two possibilities or a combination thereof.

These spectral changes were used to characterize the interactions of the two SRP protein heterodimers with the SRP RNA and with each other. The SRP9/14 heterodimer bound to the RNA much more tightly (K_d < 0.1 nM) than did the SRP68/72 heterodimer (K_d = 7 nM). SRP68/72 therefore dissociated from the RNA at a higher rate, and was exchanged from one SRP RNA to another much more quickly than SRP9/14 (Figure 6). The reason for the great disparity in the binding affinities of the heterodimers for the SRP RNA is not clear. However, it is interesting that SRP9/14, the protein which associates most tightly with the RNA, binds to a fragment containing the 60 nucleotides at the 5′ end of the SRP RNA (Strub et al., 1991). The ribosomal proteins that nucleate the assembly of the bacterial ribosomal subunits also bind near the 5′ ends of the 16S and 23S rRNAs, presumably because this portion of the rRNA is exposed first during RNA synthesis in vivo (Nowotny & Nierhaus, 1982, 1988). It is therefore possible that SRP assembly occurs in the nucleus and that the very tight binding of SRP9/14 to SRP RNA constitutes a mechanism for ensuring that SRP RNA folding and SRP assembly are initiated properly as the RNA is being synthesized.

Previous work has shown that the SRP9/14 heterodimer must form before these SRP proteins will associate with SRP RNA (Strub & Walter, 1990). Similarly, in vitro-translated SRP68 does not, by itself, bind to SRP RNA, which suggests that the SRP68/72 heterodimer must form prior to the binding of these proteins to SRP RNA (Herz et al., 1990). It is therefore conceivable that the association of the two heterodimers and the two monomers with SRP RNA may have to occur in a specific order for the assembly of a functional SRP. Yet we found that each heterodimer was able to bind to SRP RNA in the absence of the other and that the association of SRP heterodimers with SRP RNA can occur in either order to form SRP9/14·SRP RNA-Fl·SRP68/72 particles that are spectroscopically the same (Figure 7). Although SRP9/14 binds very tightly to the SRP RNA and the footprint-identified SRP9/14- and SRP68/72-binding sites on the RNA are separated by only several base pairs (Siegel & Walter, 1988b; Strub et al., 1991), the presence of SRP9/14 on the RNA did not increase the affinity of SRP68/72 for the RNA (Figure 8). There is therefore no detectable interaction between these heterodimers, either via a direct protein–protein contact or via an allosteric conformational change that is transmitted through the RNA. We conclude from these data that SRP9/14 and SRP68/72 bind independently and noncooperatively to SRP RNA in the absence of SRP19 and SRP54.

The apparent absence of cooperativity in the association of SRP9/14 and SRP68/72 with SRP RNA also suggests that the SRP9/14 domain does not interact with the SRP68/72 domain of SRP during function. Consistent with this possibility, the removal of SRP9/14 and the *Alu*-like segments of SRP RNA from the SRP yields a ribonucleoprotein complex that can still promote translocation, but is unable to arrest elongation (Siegel & Walter, 1986). The SRP domain that mediates elongation arrest and the SRP domain that mediates translocation therefore appear to be uncoupled and distinct both structurally and functionally. However, a direct or indirect interaction between SRP68/72 and SRP9/14 when the complete SRP is bound to the ribosome, signal sequence, and/or SRP receptor has not been ruled out experimentally.

In this paper, we have investigated the interactions of the SRP heterodimers with SRP RNA and with each other in the absence of the other two protein components of SRP. Previous experiments suggested that the in vitro assembly of complete SRP is cooperative (Walter & Blobel, 1983a). This conclusion was based on the observation that the total translocation and elongation arrest activity of reconstituted SRP remained constant when the amount of SRP RNA added to a constant amount of SRP proteins in a reconstitution mix was increased 8-fold (Walter & Blobel, 1983a). Walter and Blobel reasoned that if the SRP proteins bound randomly to the SRP RNA, then the 8-fold increase in RNA concentration should decrease substantially the number of RNA molecules that had a full complement of bound SRP proteins, and hence would be active, when SRP proteins exchanged randomly onto the excess of added RNA molecules. Since the fluorescence data show that SRP68/72 exchanges readily between SRP RNAs in the presence of SRP9/14, it would appear that any cooperativity in the assembly of SRP is mediated by SRP54 and/or SRP19.

Acknowledgments

We thank Bonnie Watson for doing some of the titrations, Dr. Katharina Strub for the pSP 7SL plasmid and helpful advice, Drs. Gregory D. Reinhart and David M. Jameson for doing the lifetime measurements, Dr. V. Ann Dell for preparing the anti-fluorescein antibodies, Drs. David L. Miller, William C. Merrick, and F. William Studier for gifts of purified elongation factor Tu, elongation factor 1α, and BL21/pAR1219 cells, respectively, and Dr. Olke Uhlenbeck and Jia Ye for helpful discussions.

References

Abrahamson, J. K., Laue, T. M., Miller, D. L., & Johnson, A. E. (1985) *Biochemistry 24*, 692–700.

Adkins, H. J., Miller, D. L., & Johnson, A. E. (1983) *Biochemistry 22*, 1208–1217.

Alcala, J. R., Gratton, E., & Prendergast, F. G. (1987) *Biophys. J. 51*, 587–596.

Andreazolli, M., & Gerbi, S. A. (1991) *EMBO J. 10*, 767–777.

Andrews, D. W., Walter, P., & Ottensmeyer, F. P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 785–789.

Andrews, D. W., Walter, P., & Ottensmeyer, F. P. (1987) *EMBO J. 6*, 3471–3477.

Connolly, T., & Gilmore, R. (1986) *J. Cell Biol. 103*, 2253–2261.

Connolly, T., & Gilmore, R. (1989) *Cell 57*, 599–610.

Connolly, T., Rapiejko, P. J., & Gilmore, R. (1991) *Science 252*, 1171–1173.

Davanloo, P., Rosenberg, A. H., Dunn, J. J., & Studier, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2035–2039.

Dell, V. A. (1987) Ph.D. Dissertation, University of Oklahoma.

Dell, V. A., Miller, D. L., & Johnson, A. E. (1990) *Biochemistry 29*, 1757–1763.

Erickson, A. H., & Blobel, G. (1983) *Methods Enzymol. 96*, 38–50.

Gilmore, R., Blobel, G., & Walter, P. (1982a) *J. Cell Biol. 95*, 463–469.
Gilmore, R., Walter, P., & Blobel, G. (1982b) *J. Cell Biol. 95*, 470–477.
Grodberg, J., & Dunn, J. J. (1988) *J. Bacteriol. 170*, 1245–1253.
Harlow, E., & Lane, D. (1988) *Antibodies. A Lab Manual*, pp 521–523, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Hazlett, T. L., Johnson, A. E., & Jameson, D. M. (1989) *Biochemistry 28*, 4109–4117.
Herz, J., Flint, N., Stanley, K., Frank, R., & Dobberstein, B. (1990) *FEBS Lett. 276*, 103–107.
High, S., & Dobberstein, B. (1991) *J. Cell Biol. 113*, 229–233.
High, S., Flint, N., & Dobberstein, B. (1991a) *J. Cell Biol. 113*, 25–34.
High, S., Görlich, D., Wiedmann, M., Rapoport, T. A., & Dobberstein, B. (1991b) *J. Cell Biol. 113*, 35–44.
Hoffman, K. E., & Gilmore, R. (1988) *J. Biol. Chem. 263*, 4381–4385.
Hudson, L., & Hay, F. C. (1980) *Practical Immunology*, 2nd ed., pp 1, 2, 11, Blackwell Scientific Publications, Boston, MA.
Isaacs, B. S., Husten, E. J., Esmon, C. T., & Johnson, A. E. (1986) *Biochemistry 25*, 4958–4969.
Janiak, F., Dell, V. A., Abrahamson, J. K., Watson, B. S., Miller, D. L., & Johnson, A. E. (1990) *Biochemistry 29*, 4268–4277.
Johnson, A. E., Janiak, F., Dell, V. A., & Abrahamson, J. K. (1986) in *Structure, Function, and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 541–555, Springer-Verlag, New York.
Krieg, U. C., Walter, P., & Johnson, A. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8604–8608.
Krieg, U. C., Johnson, A. E., & Walter, P. (1989) *J. Cell Biol. 109*, 2033–2043.
Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E. S., Bielka, H., & Rapoport, T. A. (1986) *Nature 320*, 634–636.
Lehrer, S. S., & Leavis, P. C. (1978) *Methods Enzymol. 49*, 222–236.
Mercola, D. A., Morris, J. W. S., & Arquilla, E. R. (1972) *Biochemistry 11*, 3860–3874.
Meyer, D. I., Krause, E., & Dobberstein, B. (1982) *Nature 297*, 647–650.
Milligan, J. F., & Uhlenbeck, O. C. (1989) *Methods Enzymol. 180*, 51–62.
Mizushima, S., & Nomura, M. (1970) *Nature (London) 226*, 1214–1218.
Nomura, M., Traub, P., Guthrie, C., & Nashimoto, H. (1969) *J. Cell. Physiol. 74*, 241–252.
Nowotny, V., & Nierhaus, K. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7238–7242.
Nowotny, V., & Nierhaus, K. H. (1988) *Biochemistry 27*, 7051–7055.
Odom, O. W., Robbins, D. J., Lynch, J., Dottavio-Martin, D., Kramer, G., & Hardesty, B. (1980) *Biochemistry 19*, 5947–5954.
Römisch, K., Webb, J., Lingelbach, K., Gausepohl, H., & Dobberstein, B. (1990) *J. Cell Biol. 111*, 1793–1802.
Scoulica, E., Krause, E., Meese, K., & Dobberstein, B. (1987) *Eur. J. Biochem. 163*, 519–528.
Siegel, V., & Walter, P. (1985) *J. Cell Biol. 100*, 1913–1921.
Siegel, V., & Walter, P. (1986) *Nature 320*, 81–84.
Siegel, V., & Walter, P. (1988a) *Cell 52*, 39–49.
Siegel, V., & Walter, P. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1801–1805.
Strub, K., & Walter, P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9747–9751.
Strub, K., & Walter, P. (1990) *Mol. Cell. Biol. 10*, 777–784.
Strub, K., Moss, J., & Walter, P. (1991) *Mol. Cell. Biol. 11*, 3949–3959.
Thrift, R. N., Andrews, D. W., Walter, P., & Johnson, A. E. (1991) *J. Cell Biol. 112*, 809–821.
Walter, P., & Blobel, G. (1981) *J. Cell Biol. 91*, 557–561.
Walter, P., & Blobel, G. (1982) *Nature 299*, 691–698.
Walter, P., & Blobel, G. (1983a) *Cell 34*, 525–533.
Walter, P., & Blobel, G. (1983b) *Methods Enzymol. 96*, 84–93.
Walter, P., & Blobel, G. (1983c) *Methods Enzymol. 96*, 682–691.
Walter, P., & Lingappa, V. R. (1986) *Annu. Rev. Cell Biol. 2*, 499–516.
Walter, P., Ibrahimi, I., & Blobel, G. (1981) *J. Cell Biol. 91*, 545–550.
Wells, B. D., & Cantor, C. R. (1977) *Nucleic Acids Res. 4*, 1667–1680.
Wiedmann, M., Kurzchalia, T. V., Bielka, H., & Rapoport, T. A. (1987) *J. Cell Biol. 104*, 201–208.
Wiedmann, M., Görlich, D., Hartmann, E., Kurzchalia, T. V., & Rapoport, T. A. (1989) *FEBS Lett. 257*, 263–268.
Wilson, C., Connolly, T., Morrison, T., & Gilmore, R. (1988) *J. Cell Biol. 107*, 69–77.
Wolin, S. L., & Walter, P. (1989) *J. Cell Biol. 109*, 2617–2622.
Zopf, D., Bernstein, H. D., Johnson, A. E., & Walter, P. (1990) *EMBO J. 9*, 4511–4517.
Zwieb, C., & Ullu, E. (1986) *Nucleic Acids Res. 14*, 4639–4657.