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Nascent polypeptide chains emerge from the exit domain of the large ribosomal subunit: Immune mapping of the nascent chain

(protein synthesis/polysomes/β-galactosidase/electron microscopy)

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ABSTRACT **The site of the nascent polypeptide chain as it leaves the ribosome has been localized on the "exit domain" of the *Escherichia coli* ribosome by using IgG antibodies directed against the enzyme β-galactosidase (EC 3.2.1.23). Thus, a functional site has been mapped on intact 70S ribosomes. The exit site is on the large subunit, approximately 70 Å from the interface between subunits and nearly 150 Å from the central protuberance, the likely site of peptide transfer. It is adjacent to the region corresponding to the rough endoplasmic membrane binding region of the eukaryotic ribosome but distant from ribosomal components participating in mRNA recognition and polypeptide elongation (*i.e.*, distant from the "translational domain"). These results, together with the protease protection experiments of others, provide evidence that the nascent protein chain probably passes through the ribosome in an unfolded, fully extended conformation.**

The ribosome, as the organelle that translates genetic messages into proteins, has roles both in the translation of the message and in the manufacture and secretion of the protein. Currently, a good deal is known about the biochemistry of ribosomal components and factors, but this knowledge is only now being integrated with that of ribosomal three-dimensional structure. Although the locations of many of the ribosomal components involved in the translation of the code are known, little is known about the location and path of the protein chain as it passes through the ribosome. Those sites functioning in translation are clustered into part of the ribosome, composing approximately two-thirds of its volume, that we have named the "translational domain." Functional sites contained in the translational domain include: the initiation factor binding sites (1–4) located in the cleft of the small subunit, the messenger binding sites located on the platform of the small subunit (3, 5–7), the peptidyltransferase and the 5S RNA located on the central protuberance of the large subunit (8–11), and proteins mediating the GTP-dependent steps of translation that are found on the L7/L12 stalk of the large subunit (12). Together, these sites define the translational domain.

In this paper, we have investigated the location of the nascent chain as it emerges from the ribosome, a function associated with protein secretion rather than the translation steps just described. Using antibodies directed against the enzyme β-galactosidase (EC 3.2.1.23) to map the exit site of the nascent protein chain, we find that it exits from the ribosome at a single region, located on the large subunit. This region is distant from the translational domain and is approximately 150 Å from the presumed site of the peptidyltransferase (8, 9). No functional site has been mapped directly on 70S ribosomes previously, and in addition the site is found on a region of the 50S subunit where no proteins or functions have been previously found. The corresponding region of the eukaryotic ribosome (13, 14), however, contains a ribosomal membrane attachment site (15). Hence, we have named this region the "exit domain." Comparison of the distance between the exit site and the peptidyltransferase center with measurements of the length of the nascent chain protected from protease digestion by free (16–18) and membrane-bound (18) ribosomes suggests that the nascent chain passes through the ribosome as an unfolded, extended polypeptide chain.

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MATERIALS AND METHODS

Cells and Polysome Preparation. Polysomes were isolated from *Escherichia coli* A324-5 (19), a mutant constitutive for the enzyme β-galactosidase. Cells were grown at 37°C in minimal medium M63 (20) containing sodium succinate at 4 g/liter, sodium citrate at 3 g/liter, and thiamin at 1 mg/ml (19). When the culture reached a density of 0.5 OD_{550} unit it was rapidly cooled by pouring it onto crushed ice at −20°C. The cells were pelleted, washed, and resuspended in 150 mM NH_4Cl/20 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$ (buffer B) and broken in a French pressure cell at 13,800 pounds per square inch (95 MPa). The extract was clarified by centrifugation at 30,000 × *g* and polysomes were pelleted through a 15–30% sucrose gradient with a cushion of 60% sucrose at the bottom in buffer A (150 mM NH_4Cl/20 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$), using a Beckman SW 50.1 rotor (245,000 × *g* for 130 min). The yield of polysomes was 5 A_{260} units per 10^{11} cells, which is in the high range (21) for the doubling time of the mutant (180 min).

Preparation of Antibodies and Reaction with Polysomes. Antibodies were produced in rabbits by an initial subcutaneous injection (in the back) of purified β-galactosidase (provided by A. Fowler and I. Zabin) and complete Freund's adjuvant. This was followed by an intramuscular boost in the thigh with incomplete Freund's adjuvant. The IgG fraction was purified by passage of the antiserum through a staphylococcal protein-A Sepharose 4B column (Pharmacia) (22). Polysomes (4 A_{260} units) were incubated with 300 μg of IgG at 0°C for 40 min. Then polysomes that had reacted with IgG were incubated at 0°C with 40 μg of RNase A (Sigma) to cleave the message. Pairs containing two β-galactosidase-bearing monosomes linked by one IgG were separated from the monosomes in a 15–30% sucrose gradient in buffer A, using either a SW 50.1 rotor (245,000 × *g* for 105 min) or a VTi 65 rotor (113,000 × *g* for 35 min). The dimer peak was passed through a Sepharose-6B column equilibrated with buffer A to remove sucrose. Ribosomes were negatively stained with 1% uranyl acetate as described (23). Electron micrographs were obtained with a Philips 400 microscope at a magnification of ×64,500.

Assay of β-Galactosidase Activity. One milliliter of 3 mM *o*-nitrophenyl galactoside in 15 mM sodium phosphate, pH 7.0, was added to 0.5 ml of sample in buffer A and incubated at 37°C. The reaction was stopped by adding 0.5 ml of 1 M sodium car-

bonate. Absorbances were measured at 420 nm with a Beckman spectrophotometer (24).

RESULTS

The *E. coli* mutant A324-5 constitutive for β-galactosidase has a number of properties that make it useful for mapping the nascent chain. Twenty percent of the total protein of this mutant is β-galactosidase (19), and the large molecular weight of the tetramer (465,000) (25) makes it easy to separate from ribosomal proteins. In addition, the large molecular weight of the monomer (116,349) (26) allows the longer polysomes, enriched for the β-galactosidase nascent chain, to be preferentially purified by differential centrifugation.

A typical polysome profile and its β-galactosidase activity are shown in Fig. 1. The absorbance has a maximum at an approximate polysome length of 12; the maximum of the activity is displaced, as expected, toward longer polysomes. The enzymatic activity associated with the β-galactosidase polysomes has been previously reported (27) and is probably due to complementation of incomplete nascent chains with previously completed molecules to form the active tetramer (28) because incomplete monomers of β-galactosidase are inactive (25). Incubation with pancreatic RNase to break the message converts the polysomes to monosomes and displaces the β-galactosidase enzymatic activity to the monosome peak (Fig. 2*A*). When the monosome fraction is purified on sucrose gradients (Fig. 3*A*), activity remains associated with the absorbance. Addition of increasing amounts of IgG antibodies directed against β-galactosidase (Fig. 3 *B*, *C*, and *D*) reduces the height of the monomer peak and a small dimer peak is formed. At low IgG concentration enzyme activity is associated with both the monomer and the heavier fractions, and at the highest IgG concentration (Fig. 3*D*) the activity is shifted almost completely to the higher peaks. At even the highest IgG concentration, the A_{256} of the monomer peak is reduced only slightly, indicating that most ribosomes do not react with the IgG. Movement of enzyme activity out of the monomer peak with increasing IgG concentrations suggests that those ribosomes that are reacting with IgG carry the β-galactosidase activity. This is evidence that the reaction is specific for the nascent chain, and not for other ribosomal components. If the IgGs were reacting with a ribosomal protein or component other than the nascent chain, then the relative enzyme activity of the monomer peak would be the same as that of the dimer peak. The final control experiment was designed to determine if β-galactosidase is bound as a nascent polypeptide chain or if it were bound through a fortuitous nonspecific adherence to the ribosome. We conclude it is bound as a nascent

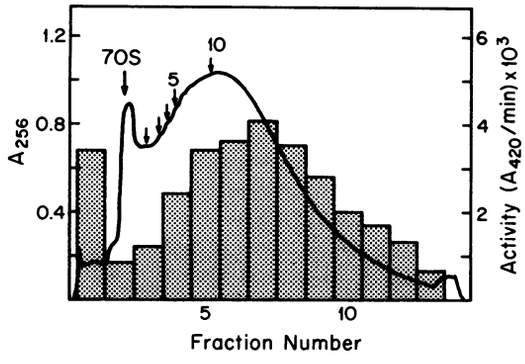

FIG. 1. Distribution of the β-galactosidase activity in polysomes. Polysomes (2.5 A_{260} units) were layered on a 15–30% sucrose gradient in buffer A and centrifuged (SW 50.1 rotor) for 30 min at 245,000 × *g*. One-fourth the volume of each fraction was assayed for β-galactosidase activity (stippled bars).

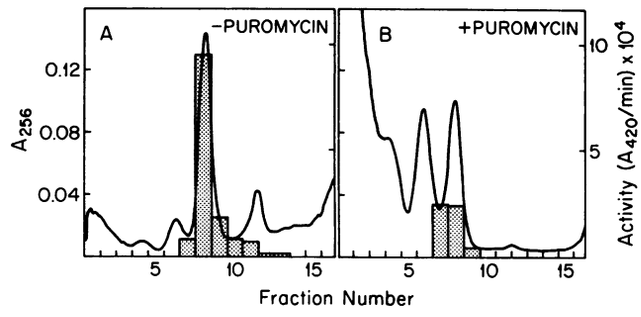

FIG. 2. The β-galactosidase activity associated with polysomes is released by puromycin. Polysomes (0.85 A_{260} unit/ml) were incubated for 8 min at 35°C in buffer A in the presence of elongation factor G at 0.6 pmol/μl and 0.1 mM GTP (*A* and *B*) plus 1 mM puromycin (*B*). The reaction mixtures were equilibrated at 0°C, and 10 μg of RNase A per A_{260} unit of ribosomes was added to each. After 1 hr at 0°C the mixtures were layered onto 15–30% sucrose gradients in buffer A. Gradients were centrifuged in a VTi 65 rotor at 113,000 × *g* for 35 min. Fractions from the gradients were assayed for β-galactosidase activity. Puromycin treatment partially dissociates the subunits. The background contributed by free β-galactosidase at the top of the gradient has been subtracted.

chain because incubation of the monosomes with puromycin and elongation factor G to release the nascent chain almost completely releases β-galactosidase activity associated with the ribosomes (Fig. 2*B*). Thus, the pair formation observed in our micrographs is dependent upon an IgG linkage through the nascent β-galactosidase chain.

Pairs of IgG-linked ribosomes were obtained for electron microscopy by allowing the longer polysomes to react with anti-β-galactosidase IgGs and then digesting the messenger between ribosomes with limited RNase treatment (as described in *Materials and Methods*). Electron micrographs of polysomes that had reacted with IgG show that the initial IgG linkage is intrapolysomal rather than interpolysomal (data not shown) and are consistent with the results of others showing that IgG against albumin nascent chain binds to polysomes without changing their sedimentation coefficient (29).

After a limited digestion with RNase (in order not to alter the ribosomal profiles observed in electron micrographs) pairs of monosomes linked through IgGs bound to their β-galactosidase

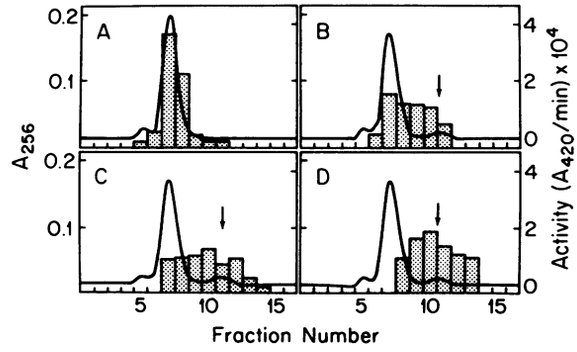

FIG. 3. Pairs of monosomes are produced by reaction with anti-β-galactosidase IgG. Polysomes (6 A_{260} units) were allowed to react with RNase A and the final mixture was separated in a sucrose gradient as in Fig. 2*A*. The monosome peak was collected from the gradient and diluted with 1 vol of buffer B. Samples with 0.3 A_{260} unit of ribosomes were allowed to react with 0, 0.6, 3, and 30 μg of IgG (*A*, *B*, *C*, and *D*, respectively) for 15 min at 0°C. The reaction mixtures were layered onto sucrose gradients and run under the same conditions as the first one. Fractions were collected and absorbance and β-galactosidase activity were measured. Arrows mark the locations of the dimer peaks.

nascent chains were purified on sucrose gradients. An electron microscopic field of this fraction is shown in Fig. 4*A*. In addition to IgG-linked pairs, shown by arrows, some disomes linked by mRNA remain. The most common views of IgG-linked ribosomes correspond to the nonoverlap projection of the ribosome (8), and examples are shown in Fig. 4 *B* and *C*. In this projection the exit site maps on the large subunit approximately 70 Å from the subunit interface. Views of ribosomes in the overlap projection (8) are relatively infrequent; however, monosomes linked through IgGs to nascent chains attached to 50S subunits are occasionally observed (Fig. 4*D*). These projections show that in the quasi-symmetric projection of the large subunit (8) the exit is on the quasi-mirror line, opposite the central protuberance. The location of the exit site in several projections of the large subunit is shown in Fig. 5.

DISCUSSION

The Exit Site Is Very Likely Distant from the Peptidyltransferase. In the *E. coli* ribosome the nascent chain emerges from the large subunit nearly 150 Å from the central protuberance of the large subunit. Several lines of evidence suggest that the central protuberance is the location of the peptidyltransferase (8, 10). In particular, protein L27 is among the proteins consistently labeled by modified aminoacyl-tRNA affinity labels bound either to the peptidyl site or to the aminoacyl site (for reviews see refs. 30 and 31; see also refs. 32–34). The location

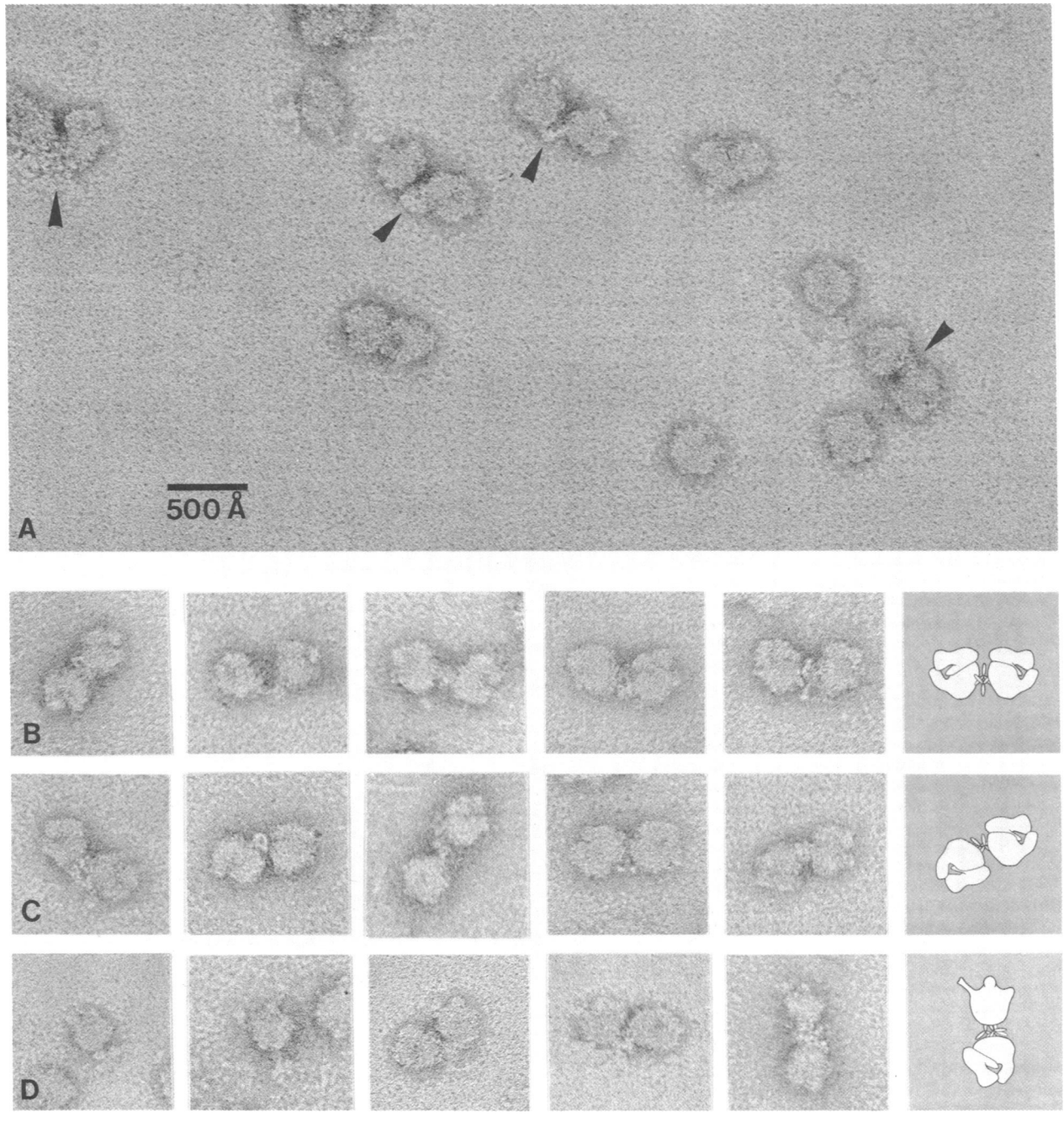

FIG. 4. Electron micrographs of ribosomes linked by antibodies against nascent chains of β-galactosidase. (*A*) A field of pairs of subunits with IgGs indicated by arrows. (*B* and *C*) Pairs of ribosomes in the nonoverlap projection connected by IgGs. The orientation of the ribosomes is shown diagrammatically in the sixth frame of each row. (*D*) Large subunits in the quasi-symmetric orientation are linked by IgG to monomeric ribosomes. No significant IgG reaction occurred with puromycin-treated ribosomes.

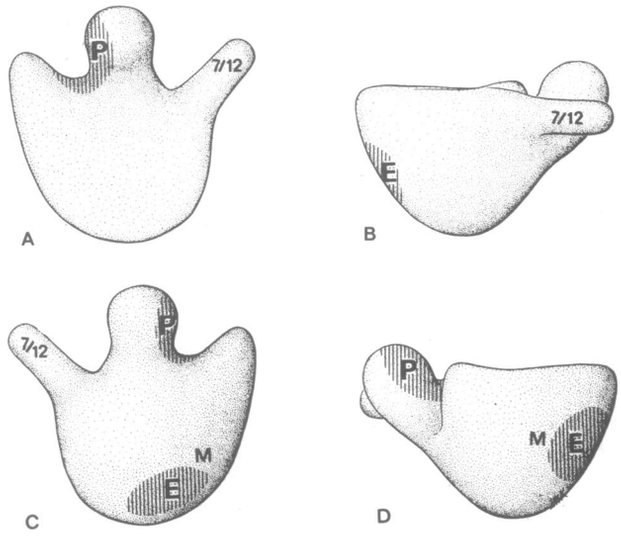

FIG. 5. Diagrammatic representation of the exit site on the surface of the large ribosomal subunit. The subunit surface contacting the small subunit is visible in *A*, is at the top of the model in *B* and *D*, and faces away from the viewer in *C*. Regions indicated are the exit site, E; the peptidyltransferase, P; the membrane binding site, M; and stalk proteins, L7/L12.

of the peptidyltransferase is shown by the letter "P" in Fig. 5 for reference. In our measurements of the distances made from electron micrographs and from the three-dimensional model of the 50S subunit (8) (assuming ± 20% uncertainty in ribosome dimensions), the transferase and the exit site are separated by 150 (±30) Å.

The Nascent Chain Is Probably Extended as It Passes Through the Ribosome. Experiments on the resistance of nascent polypeptide chains to proteolysis in eukaryotes (16, 17) and in *Bacillus subtilis* (18) have demonstrated that the carboxyl terminus of the chain is protected from degradation. This region of the chain contains the most recently synthesized 30–40 residues (30–35, 39, and 30 residues in refs. 16, 17, and 18, respectively). The "protected region" is sufficiently long to span the distance from the peptidyltransferase to the exit site but only if the chain is in an unfolded, fully extended conformation. Assuming that one extended residue is 3.6 Å long, then approximately 41 (±8) residues are needed to reach 150 (±30) Å. If the protein were in any nonextended conformation—for example, the α helix, in which each residue occupies 1.5 Å—then it could not span this distance. Hence, the protection experiments suggest that within the ribosome the chain is extended. This conclusion is not dependent on the exact site of peptidyl transfer and is valid, provided the transferase is located within the translational domain. Although the protected region in the prokaryotic ribosome (30 residues) is somewhat shorter than would have been predicted (assuming an extended chain), this effectively makes the elongated conformation more likely.

The shortest distance between the transferase and the exit site is the path directly through the large subunit. The direct path is about as far as an unextended chain of 40 residues can reach; thus a protected exit groove along the surface is less likely because this path would exceed the available chain length. Our conclusions, although very tentative, are hence more compatible with a tunnel through the large subunit. If correct, however, this model suggests that this tunnel may have some unusual properties, in addition to a small diameter, in order to prevent the folding of the nascent chain.

Transport of the nascent chain through the ribosome as an unfolded molecule has the desirable property that proteins can be transported independently of their sequences. If a ribosome is to be a *general* machine for the manufacture of proteins, then it must be able to process them independently of their conformational states. In principle, the only conformational state that is common to all proteins is the fully extended one.

The Exit Domain Is Proximal to the Membrane and the Translational Domain Faces the Cytoplasm. The earliest clues to the location of the nascent chain came from experiments (35) showing that the ribosomes are attached to membranes of the rough endoplasmic reticulum (RER) primarily through the large subunit. Subsequently it was demonstrated that eukaryotic ribosomes are attached to the membranes of the RER by two types of interactions (36). One of these is through the nascent chain and the second may involve integral membrane proteins (37, 38). The first attachment can be released by treatment with the antibiotic puromycin, indicating that it occurs through an anchoring of the nascent chain. The second is sensitive to high concentrations of monovalent salts and occurs directly between the large subunit and the membrane (see also ref. 15). In prokaryotes attachment to the plasma membrane principally occurs through the nascent chain (39). Although they differ in size, eukaryotic and prokaryotic ribosomes exhibit similar geometries and features (13, 40, 41) so that they may be compared. Because the nonoverlap profile of the prokaryotic ribosome corresponds to the "frontal view" of eukaryotic ribosomes, the membrane binding site (15) on the eukaryotic ribosome from lizard oocytes has been related to a corresponding region of the prokaryotic ribosome (14). This comparison suggests that, in the quasi-symmetric profile of the large subunit, the membrane binding region (indicated by "M" in Fig. 5) is on the side of the large subunit opposite L7/L12 and is adjacent to the exit site (indicated by "E" in Fig. 5) reported in this paper. Together these two sites compose the region we have named the exit domain. The ribosome and the orientation of the exit and translational domains with respect to the membrane are shown schematically in Fig. 6. In general, the exit domain is the region within 80 Å of the membrane. When a ribosome is bound to a membrane through its nascent chain the translational domain

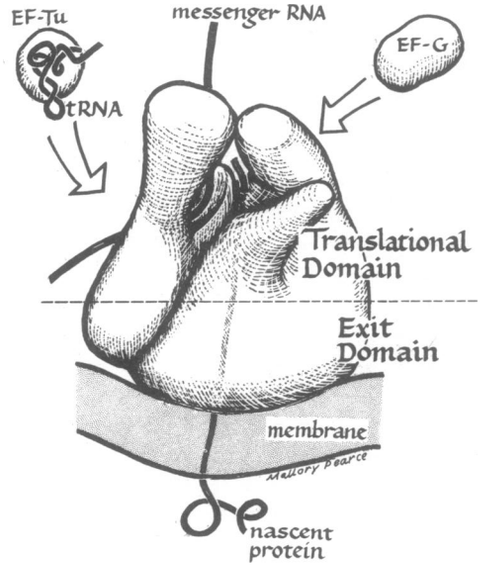

FIG. 6. Diagrammatic representation of the exit and translational domains of the ribosome and their orientations with respect to the membrane binding site. The binding sites of mRNA and elongation factors EF-Tu and EF-G are those previously inferred from the locations of ribosomal proteins (9). The nascent protein is shown as an unfolded, extended chain during its passage through the ribosome.

faces the cytoplasm. In this orientation, ligands in the cytoplasm have relatively unhindered access to sites (the messenger, the tRNA, and the factor binding sites) on the translational surface of the ribosome.

In previous studies, ribosomal functions involving translation had been found to occur on the part of the ribosomal surface named the translational domain, but no functions have been assigned for approximately one-third of the ribosomal surface. This work has localized the exit site of the nascent chain on this surface and in combination with other results has delineated the exit domain of the ribosome. The exit and translational domains are well separated on the ribosomal surface. This may reflect a functional requirement for separation, perhaps to prevent interference of the growing chain with the message and entering and exiting tRNAs and factors. It is hoped that these observations will be useful in ultimately understanding the molecular mechanisms of protein synthesis, processing, and secretion.

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