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Protein export in *Escherichia coli* requires a soluble activity

(reconstituted translation system/inverted plasma membrane vesicles/periplasmic proteins/integral membrane protein LamB/ partial purification of 12S export factor)

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ABSTRACT **By using a reconstituted cell-free system we have demonstrated the existence of a soluble activity that is required for the export of proteins in *Escherichia coli*. This export factor sediments at about 12 S. It has been partially purified by passage through an ω-NH_2-butylagarose column and by salt elution off a DEAE matrix. The export factor does not contain 6S RNA.**

In a previous report we have described a cell-free system from *Escherichia coli* that was capable of reproducing the process of protein export with fidelity (1). Among the components of this system were a membrane-free and mRNA-dependent high-speed supernatant (S-135) of an *E. coli* homogenate, plasmid DNA (to program transcription-coupled translation), and translocation-competent inverted plasma membrane vesicles (INV). Larger precursors to several exported proteins were synthesized in the absence of INV. In the co- or posttranslational presence of INV up to 25% of each of the studied precursors were translocated into the vesicle lumen (the equivalent of the periplasmic space). Most (but not all) of the translocated precursors were found to be processed by signal peptidase to their mature counterparts (1).

In this paper we describe procedures for the subfractionation of this cell-free export system and show that a soluble factor, sedimenting at 12 S, is required for the translocation of exportable proteins across the *E. coli* plasma membrane.

METHODS

Fractionation of *E. coli*. *E. coli* MRE600 cells were grown to A_{600} = 1.8 in a medium containing (per liter) 5.6 g of KH_2PO_4 (anhydrous), 28.9 g of K_2HPO_4 (anhydrous), 10 g of yeast extract, and 10 g of glucose. All subsequent manipulations were carried out between 0 and 4°C. Harvested cells were resuspended 1:1 (wt/vol) in buffer A [50 mM triethanolamine acetate, pH 7.5/50 mM KOAc/15 mM $Mg(OAc)_2$/ 1 mM dithiothreitol] containing 0.5 mM phenylmethylsulfonyl fluoride ($PhCH_2SO_2F$). This suspension was passed twice through an ice-cold French pressure cell at 4000 psi (1 psi = 6.89 kPa). An S-30 was obtained by centrifugation of the homogenate for 30 min at 30,000 × g_{av}.

The S-30 was centrifuged for 2.5 hr at 150,000 × g_{av} to yield ribosomes and a postribosomal supernatant (S-150). Both were further subfractionated.

A 1.0-ml aliquot of the S-150 was layered on 12.5 ml of a 10–30% sucrose gradient in buffer A containing 0.5 mM $PhCH_2SO_2F$. The gradients were centrifuged for 24 hr at 40,000 rpm in a Beckman SW40 rotor. Nineteen fractions identical in volume were collected. The fractions were subaliquoted, rapidly frozen in liquid N_2, and stored at −80°C for further use (Fig. 1 *A–D*).

The ribosomes were resuspended in buffer B (buffer A containing 1.0 M KOAc) in a volume equivalent to the original S-30 volume. This suspension was centrifuged for 2.5 hr at 150,000 × g_{av}. The sedimented ribosomes were resuspended in buffer B, again in a volume equivalent to the original S-30 volume. A 200-μl aliquot was layered onto 5 ml of a 10–40% sucrose gradient in buffer B. The gradients were centrifuged for 5 hr at 48,000 rpm in an SW 50.1 Beckman rotor. Gradients were then scanned at 254 nm and the major absorbance peak, representing 70S ribosomes, was collected. This material was diluted 1:5 with buffer B to lower the sucrose concentration. The ribosomes were sedimented for 12 hr at 150,000 × g_{av} and resuspended in buffer A. After centrifugation for 10 min at 12,000 × g_{av} to remove aggregates, the clear supernatant of salt-washed ribosomes (120 A_{260} units/ml) was subaliquoted, frozen in liquid N_2, and stored at −80°C.

Initiation factors (IF) were partially purified from 100 g of MRE600 cells as described (2). After salt elution from a phosphocellulose column (1.2 × 39 cm) the IF1- and IF3-containing fractions were each pooled and the IF were concentrated by adsorption and high-salt elution from small phosphocellulose columns to give final protein concentrations of 930 μg/ml (IF1 sample) and 780 μg/ml (IF3 sample), respectively. IF2 was eluted from a separate phosphocellulose column (1.2 × 39 cm). The peak fraction was used without further manipulations as the source for IF2 (approximate protein concentration, 300 μg/ml). The IF were identified by comparison to pure IF1, IF2, and IF3 (kindly provided by H. Weissbach) in polyacrylamide gel electrophoresis in Na-$DodSO_4$, and the final preparations were judged to be 30%, 90%, and 80% pure, respectively (data not shown).

Crude or gradient-purified INV (1) suspended in buffer C (50 mM triethanolamine acetate, pH 7.5/250 mM sucrose/1 mM dithiothreitol) were salt-extracted by dilution into an equal volume of buffer C containing 2 M KOAc, incubation for 5–10 min on ice, and centrifugation through a 0.5 M sucrose cushion in buffer C containing 1 M KOAc. The pelleted salt-washed INV were resuspended in buffer C to a concentration of 7–9 A_{280} units/ml, subaliquoted, frozen in liquid N_2, and stored at −80°C.

***In Vitro* Transcription.** DNA preparations of plasmid pHI-1 and *in vitro* transcription using *E. coli* RNA polymerase were performed as described (3). Plasmid pHI-1 codes for truncated β-lactamase (3) and for alkaline phosphatase, which, however, is only poorly expressed in the reconstituted system. DNA from plasmid pLB 7012B was kindly provided by S. Benson and was transcribed according to Rossi *et al.* (4). This plasmid codes for LamB.

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Abbreviations: $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; IF, initiation factor(s); SRP, signal recognition particle; INV, inverted plasma membrane vesicle(s).

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***In Vitro* Translation.** Typically, translation in the reconstituted system was performed in a 50-μl sample containing 40 mM triethanolamine acetate (pH 7.5), 140 mM KOAc, 11 mM $Mg(OAc)_2$, 20 mM NH_4OAc, 0.1 mM EDTA, 0.8 mM spermidine, 3.2% (wt/vol) polyethylene glycol 6000–8000, 2.5 mM ATP, 0.5 mM GTP, 10 mM KOH to neutralize ATP and GTP, 2 mM dithiothreitol, 8 mM phospho*enol*pyruvate, 8 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 40 μM each of 19 amino acids, 10–15 μCi of L-[^{35}S]methionine (1 Ci = 37 GBq), 200 μg of *E. coli* tRNA (Boehringer Mannheim) per ml or 125 μg of purified 4S RNA per ml, 1 μl each of the IF1 and IF3 preparations and 2 μl of the IF2 sample (see above), 7–10 A_{260} units of salt-washed ribosomes per ml, 9–10 μl of sucrose-gradient fraction 5 or 6 from the post-ribosomal supernatant (S-150) (Fig. 1*B*), and 8 μl of transcription mix. Salt-washed INV were derived from crude INV (0.36 A_{280} unit/ml) or purified INV (0.28 A_{280} unit/ml, final concentration). Incubation was at 37°C for 20 min. The reaction was stopped with 5% trichloroacetic acid or by adding proteases (see figure legends). The translation products of a 50-μl reaction were separated by electrophoresis in 10–15% polyacrylamide gradient gels in $NaDodSO_4$ and could be visualized by an overnight fluorography.

RESULTS

A soluble export factor, if it exists in *E. coli*, might resemble the eukaryotic signal recognition particle (SRP) not only in its properties (5–8) but also in its cellular distribution (9). Like SRP, such an export factor might occur (*i*) soluble in the bacterial cytosol, (*ii*) bound to ribosomes, and (*iii*) bound to the plasma membrane. One approach for detecting export factor activity in a cell-free export system is to make such a system export factor-dependent. As the export system contains cytosol and ribosomes to conduct translation and plasma membrane (in the form of INV) to accomplish translocation, it is only through subfractionation of these three components that one could hope to establish export factor dependence provided that subfractionation does not impair the system's translation activity.

The premises on which we based the methodical strategy for subfractionation were, first, that the association of the export factor with ribosomes and INV is salt dissociable (by analogy to SRP), and, second, that a soluble export factor (again, by analogy to SRP) is discernible, by virtue of a distinct sedimentation rate, from all of those components in the bacterial cytosol that are required for translation (ribosomes, translation factors, tRNA, tRNA synthetases). Based on these premises we sought to reconstitute translation from a minimal set of subcellular components that would not include export factor. Export competence would then be conferred to this reconstituted system only upon addition of a fraction containing export factor activity.

Our approach followed essentially the one described for the reconstitution of the DNA-directed synthesis of β-galactosidase from isolated components (10, 11). A 30,000 × *g* supernatant of an *E. coli* MRE600 homogenate [which differed from the S-30 previously used (1) to prepare the cell-free translation system (S-135) only by the fact that endogenous mRNA had not been read out] was subfractionated by differential centrifugation into a ribosomal pellet and a post-ribosomal supernatant (S-150). The ribosomes were subsequently washed with 1 M KOAc and recovered by centrifugation. According to a previous report (11), protein synthesis should be obtained in a system dependent on the S-150, the ribosomes, and the ribosomal salt-wash. In our hands, however, protein synthesis assayed as pHI-1-directed incorporation of L-[^{35}S]methionine into hot acid-insoluble material did not require the ribosomal salt-wash, indicating that our ribosome preparation was not completely free of those translation factors that are usually recovered from the ribosomal salt-wash. Ribosomes, therefore, were separated from contaminating proteins on a sucrose gradient in the presence of 1 M KOAc. Salt-washed ribosomes thus obtained did not restore protein synthesis to the S-150 unless the ribosomal salt-wash was added.

IF are known to be bound to ribosomes and to partition into the ribosomal salt-wash (11). Purified IF have been shown to restore translation activity to an S-150 containing salt-washed ribosomes and thus are able to substitute for the ribosomal salt-wash (11), which may also contain export factor. To avoid introducing export factor via the salt-wash, we decided to purify the IF, at least partially, by following a published procedure (2) up to the penultimate step for each IF. For the restoration of protein synthesis all three IF were required in addition to the S-150 and salt-washed ribosomes.

The postribosomal supernatant contains all of the other factors and components required for translation and is also expected to contain export factor. Therefore, the postribosomal supernatant was fractionated by sucrose gradient centrifugation (Fig. 1*A*) on the premise that all of the other factors required to reconstitute translation might be recovered in fractions separate from those containing export factor. Assaying a combination of purified ribosomes and the three IF separately with each of the 19 sucrose gradient fractions revealed that the activities necessary to reconstitute translation peaked at fractions 5 and 6 (Fig. 1*B*). The translation system consisting of purified ribosomes, partially purified IF1, IF2, and IF3, and sucrose gradient fraction 5 (or 6) therefore represented a "reconstituted" translation system.

This reconstituted system should be deficient in export factor activity. In fact, when supplemented with salt-washed INV, translocation was reduced to <5% (data not shown) when compared to the estimated 20–25% translocation in the non-reconstituted system (1).

The critical question that now arose was whether the export deficiency of the reconstituted system could be corrected by one of the gradient fractions that are not required for translation. If one of those sucrose gradient fractions could complement the export defect, our subfractionation and reconstitution approach would have accomplished its desired objective—namely, to establish an export factor-dependent assay. An indication that this was indeed the case is given by the data shown in Fig. 1*C*. The pHI-1-directed reconstituted system containing INV was assayed separately with each of the 19 sucrose-gradient fractions (a control, lane c, was assayed in the absence of a sucrose gradient fraction). Only chains exported into the vesicle lumen and therefore resistant to proteinase K digestion (1) are shown. The translocated chains comprise both the precursor (closed arrowhead) and the mature form (open arrowhead) of the truncated β-lactamase (3). When sucrose gradient fraction 9, 10, or 11 was present in the reconstituted system there was a noticeable increase in the translocated chains over background, suggesting that these fractions contain export factor activity. This activity sedimented clearly ahead of the bulk of the translation factors in fraction 5 or 6 (Fig. 1*B*). Conversely, the export factor activity sedimented slower than RNA polymerase, identifiable by the characteristic doublet of the β,β′ subunits (12) (Fig. 1*A*, closed arrows) and the groE protein complex (13) (Fig. 1*A*, open arrows). Based on these internal standards the export activity was estimated to sediment at about 12 S.

The discovery that 7S RNA was an indispensable constituent of the eukaryotic SRP (5) suggested the existence of a bacterial counterpart. 6S RNA, which is a stable, non-ribosomal RNA in *E. coli* cells, whose nucleotide sequence has been determined (14), and which was reported to exist as part of a ribonucleoprotein particle (15), was considered a likely candidate because of some sequence homology with

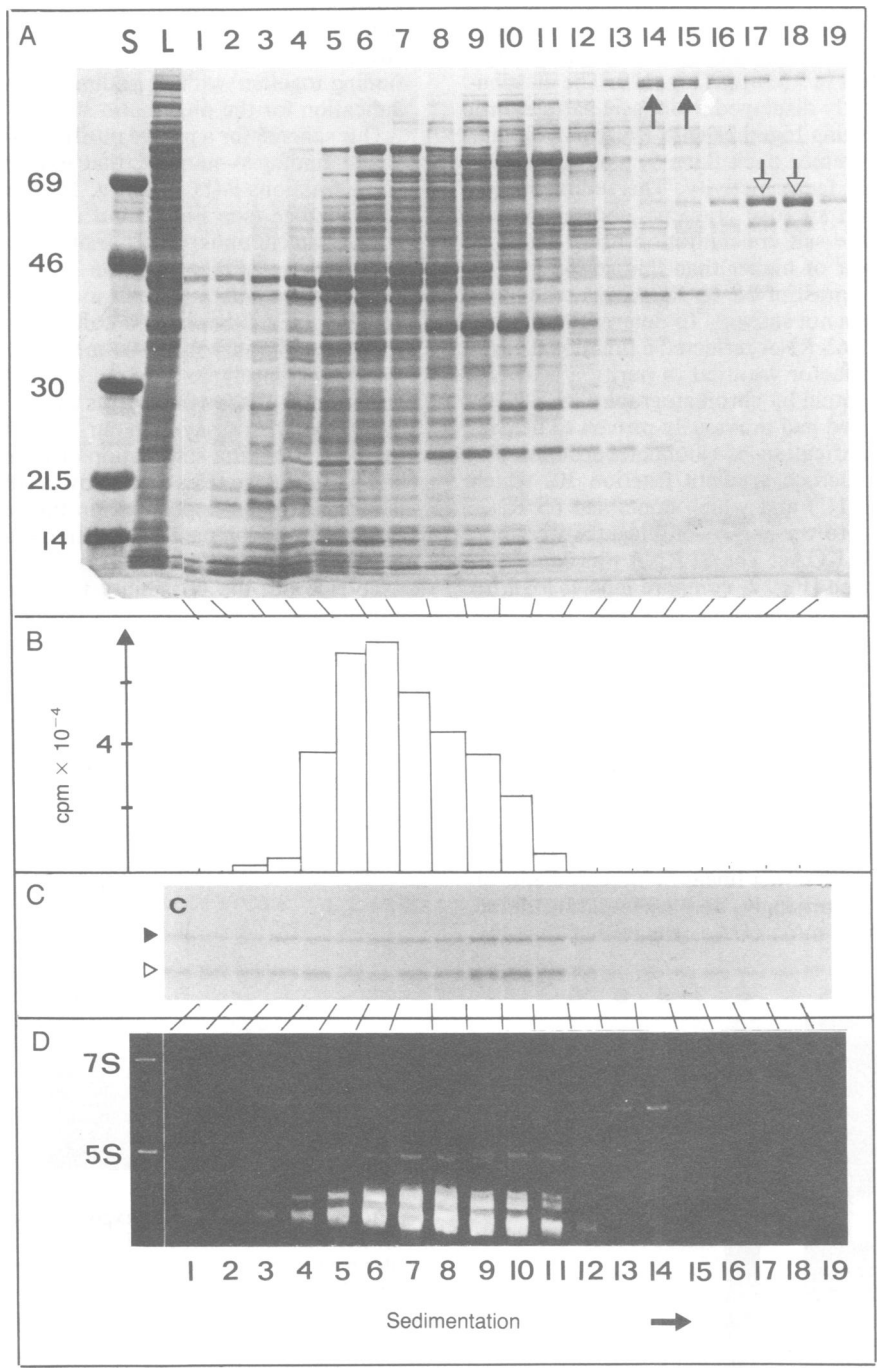

FIG. 1. Sucrose gradient fractionation of a postribosomal supernatant. (*A*) A 10-μl aliquot of each of the 19 sucrose gradient fractions (lanes 1–19), a 10-μl aliquot of the postribosomal supernatant (lane L), and molecular weight standards (lane S) were analyzed by electrophoresis in 10–15% polyacrylamide gradient gels in $NaDodSO_4$. Shown is the Coomassie blue staining pattern. The β,β' subunits of RNA polymerase in fractions 14 and 15 are indicated by upward-pointing arrows. The subunit of the groE polymer in fractions 17 and 18 is indicated by downward-pointing arrows. Numbers to the left indicate molecular mass in kilodaltons of standard proteins. (*B*) Ten-microliter aliquots of sucrose gradient fractions 1–19 were analyzed for their ability to restore translation activity to a 50-μl reaction mixture containing pHI-1 plasmid DNA, salt-washed ribosomes, and IF1, IF2, and IF3. A 3-μl aliquot of each reaction mixture was spotted onto Whatman 3MM filter papers and hot acid-insoluble radioactivity was determined. (*C*) Six-microliter aliquots of sucrose gradient fractions 1–19 were analyzed for their ability to affect translocation in a reconstituted export system (50 μl) containing pHI-1 plasmid DNA, salt-washed ribosomes, IF1, IF2, and IF3, sucrose gradient fraction 5 or 6, and salt-washed INV. In a control (lane c), buffer was added instead of a gradient fraction. Hot acid-insoluble radioactivity was determined for each reaction mixture (not shown). Aliquots containing the same amount (or slightly less) of hot acid-insoluble radioactivity as the control were then subjected to digestion with proteinase K (200 μg/ml, for 30 min at 37°C). The proteinase K-protected (i.e., translocated) polypeptides were analyzed by polyacrylamide gel electrophoresis in $NaDodSO_4$ and subsequent fluorography. The closed arrowhead points to the precursor form and the open arrowhead points to the mature form of truncated β-lactamase. Note increase of translocated chains (over background) in lanes 9–11. (*D*) RNA was extracted from 20-μl aliquots of each sucrose gradient fraction, analyzed by polyacrylamide gel electrophoresis in 7 M urea, and stained with ethidium bromide. Shown on the leftmost lane are 5S RNA and 7S RNA markers prepared as described (16).

7S RNA (16). To determine whether 6S RNA was related to the export factor activity, RNA was extracted from each of the 19 sucrose gradient fractions and separated by polyacrylamide gel electrophoresis in 7 M urea (Fig. 1*D*). The distribution of 6S RNA apparently displayed a biphasic pattern with the major peak sedimenting together with RNA polymerase (lane 14) and a second minor peak (lane 9) almost co-sedimenting with the export factor activity. The sedimentation behavior of 6S RNA on sucrose gradients, however, was strictly dependent on the salt concentration used. Thus, at salt concentrations lower or higher than that of the experiment shown in Fig. 1*D*, most of the 6S RNA was recovered from the low S peak (data not shown). To determine whether this co-sedimentation of 6S RNA reflected a functional association with the export factor we tried to purify a 6S RNA containing ribonucleoprotein by chromatography on ω-NH_2-alkylagarose. This method had previously proven to be useful for the one-step purification of ribonucleoproteins like SRP (17). Therefore, sucrose gradient fraction 10, which stimulated export (Fig. 1*C*) and which contained 6S RNA (Fig. 1*D*), was applied to an ω-NH_2-butylagarose column equilibrated with 50 mM KOAc. The 6S RNA was completely adsorbed to the column [Fig. 2, compare lane 1, load, to lane 2, flowthrough (five times as much material as on lane 1)]. The 6S RNA remained adsorbed after a wash with 50 mM KOAc (lane 3) and was eluted only at 500 mM (lane 4) and 1000 mM (lane 5) KOAc. Assaying these fractions in the reconstituted export system showed that the export factor activity was present in the 6S RNA-free flowthrough (lane 9), clearly indicating that 6S RNA is not part of the factor.

In an attempt to purify the export factor, sucrose gradient fraction 10 was passed over DEAE-Sepharose. The activity was completely bound, but could not be recovered when the column was developed with either linear or step gradients of salt (data not shown). Surprisingly, activity could be eluted with a single step of 600 mM KOAc, suggesting that, by gradually increasing the salt concentration, active components of the export factor might have been dissociated and successively eluted from the DEAE-Sepharose column. This finding together with its sedimentation behavior is a strong indication for the oligomeric structure of the export factor.

Our scheme for a partial purification took advantage of the above findings—namely, that export factor present in sucrose fractions 9–11 (see Fig. 1*C*) does not bind to ω-NH_2-butylagarose (see Fig. 2) but adsorbs to and elutes from a DEAE matrix under appropriate conditions. Thus, material from sucrose gradient fraction 10 that did not bind to ω-NH_2-butylagarose was adsorbed to a small (1/15 of the sample volume) DEAE-Sepharose column and subsequently eluted with 1.5 column vol of 600 mM KOAc. The elution buffer was immediately exchanged against the low-salt loading buffer on Sephadex G-25. This export factor-enriched preparation was then assayed in our reconstituted system. The result was a striking stimulation of translocation of both alkaline phosphatase (Fig. 3*A*, arrowheads) and truncated β-lactamase (arrows), dependent on the amount of concentrated factor added [compare no addition (lane 1) with 1 μl (lane 2) or 3 μl (lane 3) of factor].

To rule out the possibility that the observed increase in translocation merely reflected an increased rate of translation we analyzed the translation products before (Fig. 3*B*, lanes 2 and 3) and after (Fig. 3*B*, lanes 1 and 4) proteinase K digestion. The results clearly show that the rate of translation was not affected by the partially purified export factor

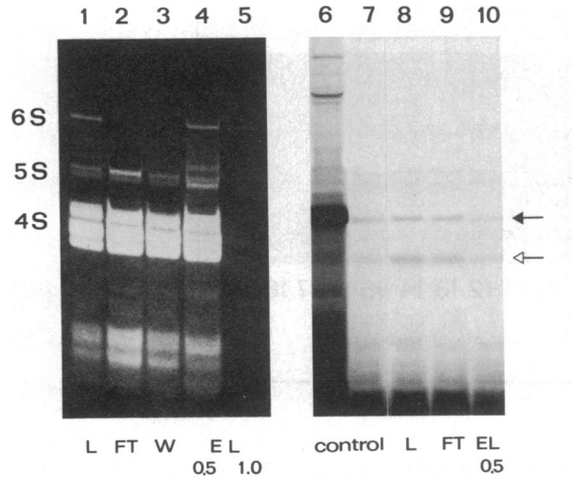

FIG. 2. Export factor does not contain 6S RNA. One milliliter of the sucrose gradient fraction 10 (see Fig. 1*C*) was passed over 200 μl of ω-NH_2-butylagarose pretreated as described (17). The flowthrough was collected and the column was washed with 3 column vol of buffer A. Bound material was eluted with 3 column vol each of 0.5 M KOAc and subsequently with 1 M KOAc in buffer A. The various effluents were analyzed for RNA (*Left*, details are as in legend to Fig. 1*D*) and translocation activity (*Right*, for experimental details see legend to Fig. 1*C*). Lane 6, total translation products. Proteinase K-resistant species of total translation products are shown in lane 7. L, load—i.e., fraction 10. FT, flowthrough. W, low salt-wash with buffer A. EL, eluate with 0.5 or 1.0 M salt in buffer A. RNA was extracted from 20 μl (L, EL), 50 μl (W), or 100 μl (FT) of sample, whereas for the activity assay 5 μl of each in a 50-μl translation mix was used.

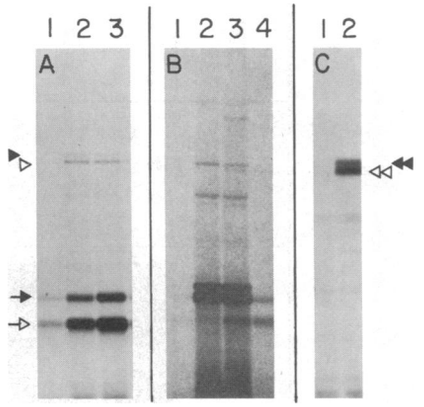

FIG. 3. Partially purified export factor stimulates translocation (*A*), not translation (*B*), and is required also for the export of an outer membrane protein (*C*). Two milliliters of gradient fraction 10 containing export factor activity (see Fig. 1*C*) was passed through an ω-NH_2-butylagarose column. The flowthrough (2 ml) was then passed over 150 μl of DEAE-Sepharose equilibrated with buffer A. After washing with buffer A, export factor was eluted with 200 μl of buffer A containing 0.6 M KOAc. The 0.6 M KOAc/buffer A was exchanged against buffer A on an 800-μl column of Sephadex G-25. (*A*) Proteinase K-treated products of a reconstituted system containing DNA of plasmid pHI-1, salt-washed ribosomes, INV, IF, sucrose gradient fraction 6, and either no added export factor (lane 1), 1 μl (lane 2), or 3 μl of partially purified export factor (lane 3). Note increase in translocated chains of truncated β-lactamase (precursor indicated by closed arrow; mature form indicated by open arrow) and of alkaline phosphatase (precursor indicated by closed arrowhead; mature form indicated by open arrowhead). (*B*) As in *A*, except for showing products *before* (lanes 2 and 3) and *after* proteinase K treatment (lanes 1 and 4); no addition (lanes 1 and 2) versus 3 μl of partially purified export factor (lanes 3 and 4). Note that export factor only stimulates translocation (lane 1 versus lane 4), not translation (lane 2 versus lane 3). (*C*) As in *A*, except that DNA of plasmid pHI-1 was replaced by DNA of plasmid pLB 7012B containing the gene for the LamB protein; lane 1, no export factor added; lane 2, 3 μl of partially purified export factor added. Precursor of LamB protein is indicated by closed double arrowheads; mature form is indicated by open double arrowheads.

(no addition of factor in lane 2; 3 μl of factor in lane 3) and that its only effect was a stimulation of translocation (compare lanes 1 and 4).

Finally, we could show that the export factor-stimulated translocation is not limited to two periplasmic proteins (truncated β-lactamase and alkaline phosphatase) but occurs for a third exported protein, the LamB protein. The latter is an integral membrane protein of the outer membrane serving as a pore protein and as a phage receptor (18, 19). As shown in Fig. 3C, there was a dramatic stimulation of translocation [or integration (1)] when concentrated factor was present in the reconstituted system [compare lane 1 (no factor added) with lane 2 (3 μl of factor added)]. If LamB were in fact integrated (1) (rather than translocated) these data would then suggest that export factor, like SRP in the case of the endoplasmic reticulum (20), is required both for translocation across and integration into the *E. coli* plasma membrane.

DISCUSSION

The data presented here deal with the detection and partial characterization of a soluble factor that is required for the export of proteins in *E. coli*.

The export factor was assayed by complementation of an export-deficient, reconstituted cell-free system that was able to conduct DNA-directed transcription-translation, that contained translocation-competent INV, but that was dependent on the addition of 12S export factor in order to translocate (or integrate) exportable proteins across (or into) the INV membranes. In constructing this factor-dependent export system we took precautions to avoid introducing factor into the system via ribosomes or INV. The latter two fractions were therefore salt-washed. This in turn necessitated the isolation of IF, as the latter occur largely bound to ribosomes (21). The other factors necessary for translation were recovered in a 4–6 S cut of a sucrose gradient-fractionated high-speed supernatant. In spite of these precautions the reconstituted system was not completely voided of export activity. Nevertheless, its endogenous export activity was low enough so that an export factor-containing fraction could signal (albeit only tenuously) its activity (Fig. 1C).

The export factor was detected in a high-speed supernatant representing the bacterial cytosol. It could be partially purified and the activity could be concentrated severalfold (Fig. 3). Its high sedimentation coefficient of 12 S and the indication we have for a reversible dissociation on a DEAE matrix suggests that it is a complex of molecules. We have shown that it does not contain 6S RNA, but it might well contain another small RNA.

We have found that an active F_1F_0-ATPase is indispensable for export to occur *in vitro* (unpublished data). However, the F_1-ATPase and the 12S export factor are clearly different. The 12S export factor, unlike isolated F_1-ATPase (unpublished data), was found not to reactivate low-salt extracted—i.e., F_1-ATPase-depleted—INV (data not shown).

Is the export factor described here related to one of the genetically defined components of the bacterial export machinery (for summary, see refs. 22 and 23)? The sec A protein was reported to fractionate as a peripheral plasma membrane protein (24). This implies a potential cytosolic localization. Moreover, the genetic identification of several different suppressors of the *sec A* mutation (25, 26; D. B. Oliver, personal communication) is indicative of a functional interaction of several gene products during protein export and, by inference, of these gene products forming a complex. Whether this complex is identical to the export factor for which we have developed an *in vitro* assay can only be answered after its purification.

We congratulate Prof. Karl Decker on the occasion of his 60th birthday. This work was supported by U.S. Public Health Service Grant GM 27155 and by a fellowship to M.M. from the Deutsche Forschungsgemeinschaft.

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