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Affinity chromatography with an immobilized RNA enzyme

(RNase P/catalytic RNA/catalytic subunit M1 RNA/C5 protein)

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ABSTRACT **M1 RNA, the catalytic subunit of *Escherichia coli* RNase P, has been covalently linked at its 3′ terminus to agarose beads. Unlike M1 RNA, which is active in solution in the absence of the protein component (C5) of RNase P, the RNA linked to the beads is active only in the presence of C5 protein. Affinity chromatography of crude extracts of *E. coli* on a column prepared from the beads to which the RNA has been crosslinked results in the purification of C5 protein in a single step. The protein has been purified in this manner from cells that contain a plasmid, pINIIIR20, which includes the gene that codes for C5 protein. A 6-fold amplification of the expression of C5 protein is found in these cells after induction as compared to cells that do not harbor the plasmid.**

Affinity chromatography has been used to purify a variety of biologically important molecules such as enzymes, cofactors, and hormone receptors (1, 2). Immobilized nucleic acids have been used to purify or identify proteins, both catalytic and noncatalytic, with which they interact. For example, ribosomal proteins that interact with 5S and 5.8S RNA have been identified by use of columns made with these rRNAs (3–5). We now report the successful preparation of a column made with an immobilized catalytic RNA, which has been used successfully to purify the protein that, together with the RNA, makes up *Escherichia coli* RNase P *in vivo*.

RNase P is the enzyme responsible for maturation of the 5′ termini of tRNA precursor molecules *in vivo* (6). In all organisms investigated thus far, it has been found that the enzyme is composed of two kinds of subunits, one of which is RNA and one of which is protein, and both of which are essential for activity *in vivo* (7). The RNA component (M1 RNA) of *E. coli* RNase P and of some other bacteria (8, 9) can, by itself, carry out the catalytic reaction under certain conditions *in vitro*. While ample quantities of these RNA molecules are available, studies of the holoenzyme complex from *E. coli* have been hampered by the difficulties in the purification of sufficient quantities of the pure protein component (C5 protein) for biochemical experimentation. The gene for this protein (*rnpA*) in *E. coli* has been cloned (10) but does not overexpress the gene product to a significant degree. We have increased our yield and facilitated preparation of C5 protein by using affinity chromatography, with immobilized M1 RNA, of cell extracts prepared from *E. coli* cells that harbor a novel subclone of the cistron and contain the *rnpA* gene adjacent to an inducible, strong promoter. In addition to C5 protein (M_r 13,700), we have identified a second protein (M_r 27,000) that binds strongly to M1 RNA.

METHODS

Bacterial Strains and Plasmids. *E. coli* strain HB101 (F^-, *hsdS20, recA13, ara14, proAZ, lacYI, galk2, rpsL20, xyl-5, mtl-1, supE44*, λ^-) was used as the host cell in the cloning experiments.

E. coli strain MRE600 was used as a source of RNase P. Cells were grown in the following media according to the required conditions for each experiment: LB medium, supplemented when necessary with ampicillin (50 μg/ml); M9 medium supplemented with glucose (2 mg/ml), leucine (50 μg/ml), proline (100 μg/ml), threonine (60 μg/ml), thiamine (1 μg/ml), and, when necessary, ampicillin (25 μg/ml).

Plasmids pINIIIompA1 [gift of M. Inouye (11); a pBR322 derivative that contains the *lpp* promoter and the inducible *lac* promoter–operator region upstream from the *ompA* gene] and pFHC1008 [donated by F. Hansen (10)] were used in the construction of the subclones. Plasmid pRR1, carrying the M1 RNA gene, has been described (12).

DNA Manipulations. Plasmid isolation and routine DNA manipulations were performed as described elsewhere (13). Restriction enzymes, Klenow fragment of *E. coli* DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Calf intestinal phosphatase was purchased from Boehringer Mannheim.

Purification of M1 RNA and RNase P. M1 RNA was purified from preparative polyacrylamide gels of total RNA from *E. coli* HB101 that contained the plasmid pRR1 as described earlier (12).

Partially purified RNase P was obtained from cellular extracts of strain MRE600 after chromatography on DEAE-Sephadex and precipitation with $(NH_4)_2SO_4$ as described (14).

Preparation of Protein Labeled with ^{35}S. Cells were grown overnight in M9 medium at 37°C, washed twice with M9 medium from which sulfate ions were omitted, and incubated for 1 hr at 37°C in the sulfate-free medium. [^{35}S]Methionine (10 μCi/ml; 1 Ci = 37 GBq) was then added, and incubation was continued for 6 more hr. For studies of the induction of cells that carry the constructed pINIIIR20 expression plasmid, 2 mM isopropyl β-D-thiogalactoside (iPrSGal) was added at the same time as the label.

Immobilization of M1 RNA. Prior to immobilization, an aliquot of M1 RNA was dephosphorylated and labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase (Promega Biotec, Madison, WI) as described (15). The labeled RNA (0.8 mg) was then oxidized with 10 mM sodium periodate (16). To examine the efficiency of oxidation, the ability of the periodate-treated M1 RNA to act as a substrate in an RNA ligase condensation with cytidine 3′,5′-[5′-^{32}P]bisphosphate was tested (17). We concluded that >95% of the M1 RNA was oxidized.

The 5′-end-labeled and oxidized M1 RNA (0.8 mg; 4×10^6 cpm, Cerenkov radiation) was coupled to 0.5 ml of agarose-adipic acid dihydrazide (P-L Biochemicals) as described (4), washed with 2 M NaCl until no further radioactivity was released, and packed in a Pasteur pipette. About 90% of the RNA was actually coupled to the gel as estimated from the radioactivity recovered in the washes.

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Abbreviations: iPrSGal, isopropyl β-D-thiogalactoside; bp, base pair(s).

Subcloning of the *rnpA* Gene that Codes for C5 Protein. The expression plasmid pINIIIR20 was constructed to increase the production of C5 protein. The inducible expression vector pINIIIompA1 (11) was cut at the unique *Bam*HI site, and the staggered ends were filled in by polymerization with the Klenow fragment of *E. coli* polymerase I and dephosphorylated with calf intestinal phosphatase.

Plasmid pFHC1008 (10), which contains part of the *rpmH* operon and includes the complete sequence of *rpmH* (the gene for ribosomal protein L34) and *rnpA* (the gene for C5 protein), was cut at the unique *Bss*HII and *Ava* I sites, and a 776-base-pair (bp) fragment that contained the *rpmH* and *rnpA* genes was purified. This fragment was cut with *Rsa* I to give four fragments (538, 142, 67, and 33 bp). The 538-bp fragment contained most of the *rpmH* gene and the complete *rnpA* gene. The staggered ends were filled with the Klenow fragment, and the mixture of fragments was ligated to the linearized vector with T4 DNA ligase. Transformants were selected by ampicillin resistance, and those carrying the 538-bp fragment in the right orientation were identified by restriction analysis. In this construct the *rpmH* gene is in phase with the coding sequence of the signal peptide of the OmpA protein (11).

When selection of transformants was carried out in LB medium, all of the clones that contained the 538-bp fragment had the fragment inserted in the wrong orientation. However, when selection was carried out in M9 medium, the right construction was obtained. This result suggests that the expression of the carboxyl-terminal-modified L34 protein does not permit viability. Leaky induction of the *lac* gene promoter by chemical contaminants in LB medium (18) is avoided in minimal medium. Note also that *E. coli* strain HB101 carrying pINIIIR20 cannot grow in the presence of 2 mM iPrSGal.

Purification of C5 Protein. *Extraction of RNAs*. The RNAs present in S30 extracts or in RNase P, partially purified at least through the DEAE-Sephadex step (14), were extracted with LiCl/EDTA by the method of Dijk and Littlechild (19) as used for preparation of active yeast ribosomal proteins (20). (We also have used LiCl/EDTA to elute basic proteins from immobilized RNA as is described below.) One volume of extract was mixed with 2 volumes of 10.5 M LiCl, and EDTA (pH 8.0, adjusted with NaOH) was added to a concentration of 10 mM. The mix was gently agitated overnight at 4°C and centrifuged at 100,000 × *g* for 10 hr. The supernatant, containing the proteins, was dialyzed against buffer A (50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/100 mM NH_4Cl/10 mM 2-mercaptoethanol).

Affinity chromatography on immobilized M1 RNA. The dialyzed supernatant from the previous step was loaded on the affinity column, and the sample was recirculated through the column overnight by a peristaltic pump. The column was then washed and eluted with buffer A that contained different concentrations of NH_4Cl as indicated in the legends to Figs. 2 and 3. C5 protein was eluted in the final step with 7 M LiCl/10 mM EDTA, pH 8.0.

Electrophoresis of Proteins. Proteins were analyzed on 0.1% $NaDodSO_4$/4 M urea/12.5% acrylamide gels by the method of Laemmli (21). Total cellular proteins were analyzed from a cell pellet (from 0.5 ml of culture) that was boiled for 3 min in loading buffer and then loaded directly on the gel. Other protein fractions were precipitated with trichloroacetic acid in the presence of 15 μg of bovine serum albumin, when necessary, before boiling in loading buffer and loading. Protein concentrations were determined by the method of Lowry *et al.* (22) with bovine serum albumin as a standard.

Assays for RNase P Activity. Reconstitution assays were performed by the direct mixing method (8) in 50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/100 mM NH_4Cl. PEG (5% wt/vol) was added where indicated. M1 RNA activity was assayed in 50 mM Tris·HCl, pH 7.4/100 mM $MgCl_2$/100 mM NH_4Cl/5% PEG. The precursor to *E. coli* $tRNA^{Tyr}$ was used as substrate (23) in all of the experiments reported here. All incubations were carried out at 37°C for the times indicated. The reaction products were analyzed on 10% (wt/vol) polyacrylamide gels as described elsewhere (8), and the amount of product formed was estimated by scanning densitometry of autoradiographs. When immobilized M1 RNA was assayed, its concentration was estimated from the radioactivity of its 5'-end label, determined by scintillation counting.

RESULTS

Properties of the M1 RNA Column. The 3' terminus of M1 RNA can be altered in various ways with little or no effect on the catalytic activity of the RNA (9, 24). We verified (data not shown) that M1 RNA from *E. coli*, oxidized with periodate to create 2' and 3' keto groups, has similar activity to that of intact M1 RNA, just as Marsh and Pace (17) found for the RNA component of *Bacillus subtilis* RNase P. The oxidized M1 RNA was coupled through a dihydrazide adipic acid spacer to agarose beads as described, and a slurry of the resulting material was tested for RNase P activity. We chose the particular beads and spacer to reduce problems of steric hindrance.

When the M1 RNA–bead complex was assayed for activity (Fig. 1), the catalytic activity was almost fully recovered in the reconstitution assay with C5 protein (lanes 5 and 6) but was almost completely lost when tested under conditions where the free RNA exhibited activity by itself (lanes 2 and 3). No activity was observed when C5 protein or M1 RNA was assayed alone in buffer containing 10 mM $MgCl_2$. Furthermore, estimates of relative rates of cleavage were made from more extensive kinetic studies (data not shown). When free and immobilized M1 RNA were assayed in the presence of C5 protein, they had about the same activity. But about 5% of the expected activity of free M1 RNA was recovered when the slurry of immobilized RNA was assayed in the absence of C5 protein. However, when the slurry was analyzed by gel electrophoresis, about 5% of the RNA ran as free M1 RNA in the gel, while the rest did not enter the gel. From this result, we infer that a small amount of M1 RNA was leached off the beads and could account for the catalytic activity detected in the absence of protein. However, we cannot rule out the possibility that this small amount of

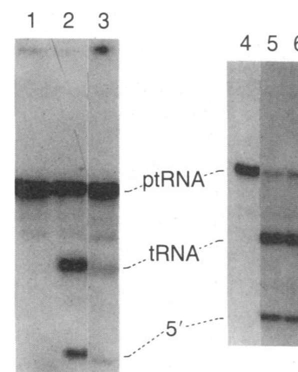

FIG. 1. Comparison of the activity of immobilized M1 RNA with that of free oxidized M1 RNA. Assays were carried out as described in the presence of 5% PEG. ptRNA, tRNA, and 5' indicate the positions of the substrate tRNA precursor molecule and the two cleavage products, respectively. Lanes: 1 and 4, no addition; 2, 10 ng of oxidized M1 RNA assayed for 15 min in buffer that contained 100 mM $MgCl_2$; 3, 10 ng of immobilized M1 RNA assayed as in lane 2; 5, 10 ng of oxidized M1 RNA assayed for 5 min in buffer that contained 10 mM $MgCl_2$ and an excess of purified C5 protein; 6, 10 ng of immobilized M1 RNA assayed as in lane 5.

activity was, in fact, due to the immobilized RNA. Analysis of the slurry after the reconstitution assay indicated that the presence of the C5 protein did not induce the release of RNA from the beads (data not shown). Therefore, the catalytic activity detected in the assay in the presence of C5 protein was due, in fact, to the coupled RNA–bead complex. We conclude that immobilization of the 3′ terminus of M1 RNA interferes with a critical step in the activation of the enzyme, in the absence of the protein cofactor. However, the RNA coupled to the beads appears to retain the desired specificity for the binding of C5 protein so that the column can be used for the successful purification of C5 protein.

To analyze the conditions that allow the purification of C5 protein on the affinity column, we used a protein mixture prepared from RNase P that had been partially purified through the DEAE-Sephadex step and $(NH_4)_2SO_4$ precipitation as described by Stark *et al.* (14). The RNAs present in this preparation were extracted by treatment with 7 M LiCl/10 mM EDTA. The resulting protein mixture was essentially free of RNA (<1%, as determined by dotting a sample on agarose gel that contains ethidium bromide and visualizing the material with a UV transilluminator). No RNase P activity was manifested by this material unless exogenous M1 RNA was added. The C5 protein in the preparation did not bind to DEAE-Sephadex (data not shown) in contrast to the holoenzyme, which does bind as a consequence of its RNA content. These results confirmed that the LiCl/EDTA extract was free of M1 RNA, and the C5 protein contained in it was still active in the RNase P reconstitution assay. Although protein extracts of *B. subtilis* RNase P that gave RNase P activity in the reconstitution assay have been made using acetic acid to remove RNA (25), this method has not yielded preparations of C5 protein from *E. coli* RNase P that are active in reconstitution assays.

A dialyzed extract of RNase P from which the RNA had been removed by treatment with LiCl/EDTA was loaded on the affinity column, and the proteins that were eluted from the immobilized RNA are shown in Fig. 2. The position of C5 protein in the PAGE analysis of the eluates is shown by the arrow adjacent to lane 16. (Note that C5 protein has an anomolous electrophoretic mobility. It migrates more slowly than expected from its molecular weight.) Optimized conditions for the binding of this protein to the column include recycling of the load sample and the use of PEG in the loading buffer. PEG increases binding efficiency by enhancing the effective local concentration of the protein.

The protein composition of the load sample and the flow-through fraction are shown in Fig. 2, lanes 2 and 3. There is some smearing of these samples because of the presence of PEG in the buffer. Proteins in subsequent eluates from the column are shown in lanes 5–15. Many proteins were bound to the column, but most were eluted with buffer that contained 0.37 M NH_4Cl (lanes 5–8). A protein of apparent M_r 27,000 bound strongly to the column but could be separated from C5 protein by elution with buffer that contained 0.7 M NH_4Cl (lanes 11–13). After this elution, only C5 protein remained bound to the column, but it could be eluted along with almost all material that is active in the RNase P reconstitution assay by 7 M LiCl/10 mM EDTA (lanes 14 and 15). This final elution step has been adapted successfully from the method used to separate ribosomal proteins from rRNA (19). Each fraction was assayed for RNase P activity in the absence and presence of M1 RNA in buffer that contained 10 mM $MgCl_2$. The only column fraction exhibiting significant activity in the presence of added M1 RNA was the one corresponding to that seen in lane 14 of Fig. 2 (LiCl/EDTA wash).

The protein species eluted from the column that has been labeled as "C5 protein" comigrates with active C5 protein purified by a different (and less efficient) technique (8). This species, eluted in the final step (Fig. 2, lane 14), was active in the reconstitution assay for RNase P activity, and so we concluded that it is, indeed, C5 protein. However, a species with similar mobility in the gels, was eluted at many of the lower concentrations of NH_4Cl but did not manifest activity in the reconstitution assay with M1 RNA. There were many basic proteins in addition to C5 protein in these eluates that could compete for binding sites on M1 RNA. It is also possible that the C5 protein in these fractions was irreversibly denatured. We have not recovered active C5 protein from the protein eluted at a lower concentration of NH_4Cl, by dialysis against buffer A, or by treatment of these fractions with LiCl/EDTA prior to dialysis. When fractions that were eluted at 0.37 M NH_4Cl were dialyzed and recycled through the column, some of the M_r 27,000 protein and the apparent C5 protein were eluted at higher salt concentrations, though most of the material still was eluted at 0.37 M. Virtually no active C5 protein was recovered. These data indicate that the apparent C5 protein that was eluted at the lower salt concentrations may indeed be denatured or may be inhibited in the reconstitution assay by the concentrations of other basic proteins, including the M_r 27,000 protein, which are relatively high compared to those found in crude extracts.

Twenty grams of *E. coli* yielded about 5.4 mg of LiCl extract and, finally, about 5 μg or about 15% of the total C5 active protein in the original 7 M LiCl/10 mM EDTA extract.

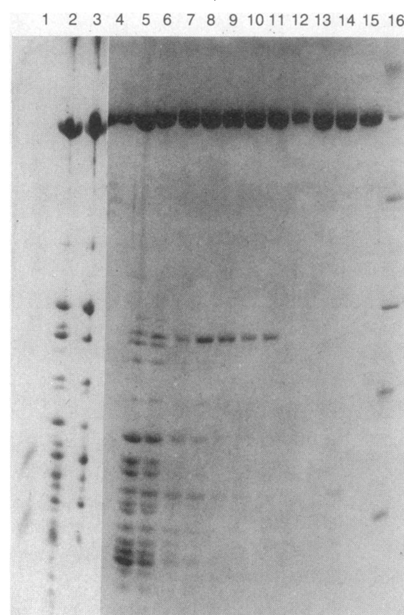

FIG. 2. Electrophoretic analysis of the fractions eluted from the affinity column. Protein extracted with LiCl/EDTA from partially purified RNase P (see text) was placed in buffer A that contained 5% PEG, and 12 ml (6 mg) was loaded on a 0.5-ml column containing 700 μg of bound M1 RNA. The sample (lane 2) was recirculated on the column for 15 hr at a flow rate of 6 ml/hr. The flow-through fraction was collected (lane 3), and the column was washed in succession with 15 ml of buffer A that contained 5% PEG (not shown), 2 ml of buffer A (lane 4), three times with 0.6 ml of 0.37 M NH_4Cl in buffer A (lanes 5–7), three times with 0.6 ml of 0.5 M NH_4Cl in buffer A (lanes 8–10), three times with 0.6 ml of 0.7 M NH_4Cl in buffer A (lanes 11–13), and twice with 0.4 ml of 7 M LiCl/10 mM EDTA (lanes 14–15). Lane 1 contains myoglobin (M_r 17,200) and a smaller contaminant. Lane 16 contains the following molecular weight markers: phosphorylase B (M_r 92,500), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400). The uppermost dense band in lanes 2–15 is the bovine serum albumin used as carrier in the preparation of the sample. Lanes 2 and 3 contained one-fifth as much sample as lanes 4–15. The arrow next to lane 16 indicates the position of C5 protein. The gel was stained with Coomassie brilliant blue.

The recovery of active C5 protein with this method was at least three times better than that obtained by preparative electrophoresis of the same amount of starting material (14), and the purification was achieved in less than half the time needed for the latter method. It is difficult to estimate accurately the extent of recovery of C5 protein because reconstituted RNase P activity depended on the concentration of added C5 protein in a nonlinear fashion (unpublished data), and pure C5 protein was inactivated on dilution. The latter problem could be overcome, in part, by inclusion of PEG in the dilution buffer. Thus, our estimates of total recovered activity may be accurate only within a factor of 2.

If one assumes that at least one C5 protein molecule can bind to one M1 RNA molecule, the capacity of the M1 RNA column is 0.11 μg of C5 protein per μg of M1 RNA available for binding. In the case we describe, the theoretical capacity is 77 μg of C5 protein. The only limitation in scaling up this procedure is the availability of M1 RNA, and that is no longer a serious obstacle.

Amplification of C5 Protein *in Vivo*. To enhance the yield of C5 protein from crude extracts of cells, we subcloned, next to a strong, inducible promoter in pINIIIompA1, the gene that codes for C5 protein (*rnpA*) and the adjacent gene that codes for the ribosomal protein L34 (*rpmH*) from pFHC1008. Crude extracts were made from cells that harbored the new vector, pINIIIR20, and from the parent cells with no vector. These extracts were both subjected to electrophoresis for analysis of the protein content and also were passed over the M1 RNA column.

The production of proteins in cells with pINIIIR20, after induction with iPrSGal, is shown in Fig. 3, lane 2. Lane 1 shows proteins from an extract of uninduced cells. Note the massive increase in the production of a protein that corresponds in mobility to L34, the gene that is adjacent to and upstream from *rnpA*, the gene that codes for C5 protein. The upper arrow in lane 2 points to a faint minor band, unseen in

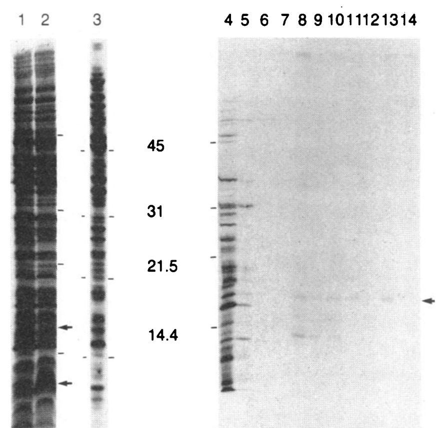

FIG. 3. Autoradiogram of the electrophoretic analysis of proteins of [^{35}S]methionine-labeled *E. coli* strain HB101 that carries pINIIIR20. Lanes: 1, uninduced cells; 2, cells induced with 2 mM iPrSGal; 3, S30 extract prepared from induced cells; 4–14, elution patterns from the immobilized M1 RNA column of the S30 extract shown in lane 3 after extraction of RNAs with 7 M LiCl/10 mM EDTA (elution was done as in Fig. 2, but the 0.7 M NH_4Cl washes were omitted); 4, flow-through fraction; 5, buffer A containing 5% PEG wash; 6, buffer A wash; 7–9, 0.37 M NH_4Cl washes; 10–12, 0.5 M NH_4Cl washes; 13–14, 7 M LiCl/10 mM EDTA washes. Gels were dried and exposed for 24 hr (lanes 1–2), 1 wk (lane 3), or 4 wk (lanes 4–14). The arrows next to lane 2 indicate the positions of the L34 fusion protein (lower arrow) and C5 protein (upper arrow). The hatch marks next to lanes 2, 3, and 4 show the positions of the molecular weight ($\times 10^{-3}$) markers between lanes 3 and 4. The arrow next to lane 14 indicates the position of the C5 protein. Note that ribosomal protein L34 is not present in the preparation of the S30 extract (lane 3) from the total cell protein (lane 2).

the uninduced cells, which has the mobility of C5 protein. This analysis did not allow us to estimate the amount of amplification of production of C5 protein. Therefore, we treated the crude extract of pINIIIR20 with 7 M LiCl/10 mM EDTA to remove RNA and used the resulting protein in reconstitution assays for RNase P activity. The resultant activity of RNase P from the crude extracts of *E. coli* strain HB101 harboring plasmid pINIIIR20 induced with iPrSGal is about 6-fold higher than the activity in the extract from uninduced cells. We presumed, therefore, that the production of C5 protein in the induced cells is at least 6-fold greater than in uninduced cells, and this presumption was verified by experiments with radioactively labeled extracts.

When an S30 cell extract, from which RNA had been removed with 7 M LiCl/10 mM EDTA (Fig. 3, lane 3), of induced cells harboring pINIIIR20 was passed over the immobilized M1 RNA column, >80% of the protein found in the column fraction eluted with 7 M LiCl/10 mM EDTA (Fig. 3, lane 13) was C5 protein, as judged by autoradiography of material labeled with ^{35}S. The yield of C5 protein in this fraction is 4- to 6-fold greater than that found in extracts of uninduced cells as determined by comparisons of the recovery of ^{35}S-labeled C5 protein. The M_r 27,000 protein was not apparent in the material eluted with 7 M LiCl from the column that was derived from the induced cells. Our results demonstrate that it is possible to purify a very minor protein from *E. coli* (about 250 copies per cell) in one step on the immobilized M1 RNA column.

DISCUSSION

Affinity chromatography of proteins on immobilized RNAs has been used as an analytical tool to determine which proteins interact in a specific manner with different RNAs (3–5). We have used this method, not only to purify proteins that bind strongly to M1 RNA, a catalytic macromolecule, but also to prepare these proteins in amounts useful for biochemical experimentation. In fact, we have been able to purify C5, the protein component of RNase P, in a single chromatographic step from a crude extract of *E. coli*. We also have found another protein of M_r 27,000 that binds strongly to M1 RNA. In addition, the method we have developed allows protein purification to be achieved without the use of denaturing agents such as $NaDodSO_4$, urea, or acetic acid or of lyophilization. Although the high concentrations of salts we used to separate RNA from protein in the initial crude extract and in the column elution buffers in general will affect the tertiary structure of proteins, the recovered protein is in a "native" state (see ref. 19). The LiCl/EDTA method has been used to extract yeast ribosomal proteins that are subsequently able to reconstitute functional 60S yeast ribosomal subunits (20). The C5 protein we purified is active in the reconstitution assay for RNase P activity and, thus, is highly suitable for further studies of the mechanism of action of RNase P.

At present we do not know if the M_r 27,000 protein is related to the function of M1 RNA or the processing of the precursor to M1 RNA *in vivo*. Although the size of the M_r 27,000 protein may be compatible with that of a dimer of C5 protein, it is not likely that such a dimer survives boiling in $NaDodSO_4$ or electrophoresis through $NaDodSO_4$ and urea, both procedures being part of our protocols. In addition, the M_r 27,000 protein is not seen in the final column fractions of ^{35}S-labeled extracts of HB101 cells that do or do not harbor pINIIIR20, although large amounts of C5 protein are observed. This observation indicates that the M_r 27,000 protein lacks methionine residues and, therefore, is unrelated to C5 protein.

The elution from the affinity column of C5 protein over a wide range of salt concentrations is not unexpected because

we know that M1 RNA can exist in a variety of conformations, some of which may provide higher specificity for binding of C5 protein (and M_r 27,000 protein) than others. However, the amount of active C5 protein recovered in these fractions is not well correlated with the apparent amount of protein, most of the activity being recovered in the final wash with 7 M LiCl/10 mM EDTA. At this point we cannot say for certain whether the species that comigrates with C5 protein is inactive or modified C5 protein, which binds less strongly to M1 RNA than does native C5 protein, nor do we know whether its activity is simply inhibited by the presence of basic proteins that copurify with it.

The amount of C5 protein in cellular extracts has been increased by subcloning a cistron that contains the gene coding for this protein (*rnpA*) from the plasmid pFHC1008 to a site adjacent to an inducible, strong promoter in the plasmid pINIIIompA1. Normally, *rnpA* is cotranscribed with the gene for ribosomal protein L34 (*rpmH*) and two other proteins (10), but no significant overproduction of the *rnpA* gene product is observed in cells harboring pFHC1008, although L34 is amplified (10). When these two genes are placed adjacent to the *lpp* and *lac* promoters in pINIIIR20, an ≈6-fold amplification of C5 protein is observed, as judged by the amount of C5 recovered after affinity chromatography. Thus, a combination of the novel column technique and genetic engineering has led to a significant improvement in the yield and efficiency of purification of C5 protein.

In constructing the immobilized RNA column, we took advantage of the fact that the 3′ terminus of M1 RNA can be altered in various ways without significantly diminishing the function of the molecule (24). Furthermore, the length of the bridge to the agarose beads was chosen to minimize steric problems and, thus, we anticipated that the immobilized RNA would still retain its catalytic function. However, catalytic activity at the same rate as shown by the equivalent amount of free RNA is detected only in the presence of the protein cofactor. This result prompts the speculation that, for the RNA to function by itself, it must have some degree of conformational flexibility at its 3′ terminus that is absent when it is covalently linked to the beads. Conformational change, such as dimerization (26), as a prelude to catalytic function may be slowed or prevented when M1 RNA is linked to the beads. Indeed, some immobilized protein enzymes have been reported to undergo conformational changes 10^3–10^5 times more slowly than do the free proteins (27). For the immobilized M1 RNA to become active, through whatever conformational change may be needed, C5 protein must be present as an essential cofactor. The requirement for a protein cofactor in this reaction bears on speculation regarding early biochemical systems. If the first enzymes were composed of RNA and had properties similar to those of M1 RNA, immobilization on the interior of primitive cell membranes or in simple organelles would have created immediate selective pressure for the participation of proteins in enzymatic reactions. The preparation of enzymatically active immobilized RNA may also be of practical significance since catalytically active immobilized proteins have found widespread applications (28).

Finally, we note that active RNase P can be reconstituted from M1 RNA and the appropriate protein components from HeLa cells (29) and *B. subtilis* (8). These observations raise the possibility that the affinity-chromatography procedure we describe here may be useful in purifying the protein components of RNase P from a variety of organisms. The procedure also may be adapted to the purification of protein cofactors that stimulate other reactions known to be catalyzed by RNA (30) or in which ribonucleoproteins are intimately involved (31).

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1. Lerman, L. S. (1953) *Proc. Natl. Acad. Sci. USA* **39**, 232–236.
2. Wilchek, M., Miron, T. & Kohn, J. (1984) *Methods Enzymol.* **104**, 3–55.
3. Burrell, H. R. & Horowitz, J. (1977) *Eur. J. Biochem.* **75**, 533–544.
4. Ulbrich, N. & Wool, I. G. (1978) *J. Biol. Chem.* **253**, 9049–9052.
5. Ulbrich, N., Lin, A. & Wool, I. G. (1979) *J. Biol. Chem.* **254**, 8641–8645.
6. Altman, S., Guerrier-Takada, C., Frankfort, H. & Robertson, H. D. (1982) in *Nucleases*, eds. Linn, S. & Roberts, R. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 243–274.
7. Kole, R., Baer, M., Stark, B. C. & Altman, S. (1980) *Cell* **19**, 881–887.
8. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. (1983) *Cell* **35**, 849–857.
9. Guerrier-Takada, C. & Altman, S. (1984) *Science* **223**, 285–286.
10. Hansen, F. G., Hansen, E. B. & Atlung, T. (1985) *Gene* **38**, 85–93.
11. Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. & Inouye, M. (1984) *EMBO J.* **3**, 2437–2442.
12. Reed, R. E., Baer, M. F., Guerrier-Takada, C., Donis-Keller, H. & Altman, S. (1982) *Cell* **30**, 627–636.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
14. Stark, B. C., Kole, R., Bowman, E. J. & Altman, S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3717–3721.
15. Guerrier-Takada, C. & Altman, S. (1984) *Biochemistry* **23**, 6327–6334.
16. Fahnestock, S. R. & Nomura, M. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 363–365.
17. Marsh, T. L. & Pace, N. R. (1985) *Science* **229**, 79–81.
18. Takahara, M., Hibler, D. W., Barr, P. J., Gerlt, J. A. & Inouye, M. (1985) *J. Biol. Chem.* **260**, 2670–2674.
19. Dijk, J. & Littlechild, J. (1979) *Methods Enzymol.* **59**, 481–502.
20. Vioque, A. & Palacian, E. (1985) *Mol. Cell. Biochem.* **66**, 55–60.
21. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
23. Robertson, H. D., Altman, S. & Smith, J. D. (1972) *J. Biol. Chem.* **247**, 5243–5251.
24. Guerrier-Takada, C. & Altman, S. (1986) *Cell* **45**, 177–183.
25. Gardiner, K. J., Marsh, T. L. & Pace, N. (1985) *J. Biol. Chem.* **260**, 5415–5419.
26. Guerrier-Takada, C., Haydock, K., Allen, L. & Altman, S. (1986) *Biochemistry* **25**, 1509–1515.
27. Davydov, R. M., Vesterman, B. G., Genkin, M. V., Blumenfeld, L. A. & Krylov, O. V. (1981) *Dokl. Akad. Nauk. SSSR* **256**, 491–494.
28. Mosbach, K., ed. (1976) *Methods Enzymol.* **44**, 491–936.
29. Gold, H. A. & Altman, S. (1986) *Cell* **44**, 243–249.
30. Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E. & Cech, T. R. (1982) *Cell* **31**, 147–157.
31. Cech, T. R. & Bass, B. L. (1986) *Annu. Rev. Biochem.* **55**, 599–629.