Proc. Nat. Acad. Sci. USA
Vol. 71, No. 2, pp. 446–450, February 1974

In Vitro Synthesis of Ribosomal Proteins Directed by *Escherichia coli* DNA*

(ribosome reconstitution/radio-immunodiffusion/protein identification)

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Communicated by James F. Crow, September 17, 1973

ABSTRACT ***In vitro* synthesis of a number of *E. coli* 30S ribosomal proteins has been demonstrated in a cell-free system consisting of ribosomes, initiation factors, RNA polymerase, a fraction containing soluble enzymes and factors, and *E. coli* DNA. DNA-dependent synthesis of the following 30S proteins has been demonstrated: S4, S5, S7, S8, S9, S10, S13, S14, S16, S19, and S20.**

The regulation of biosynthesis of ribosomes, as well as individual ribosomal components, is an important aspect of the regulation of cellular growth. Bacterial ribosomes contain three kinds of rRNAs and 50–60 different proteins (r-proteins). While considerable information about the regulation of rRNA synthesis has been obtained, little is known of the regulation of synthesis of r-proteins (for reviews, see refs. 1–3).

Investigation of the mechanism and regulation of r-protein synthesis would be simplified if one could synthesize r-proteins *in vitro* in a system that couples transcription of r-protein genes with translation of the messenger transcripts. We now report development of a system capable of synthesis of a number of r-proteins. The identification of several r-protein products and the evidence for DNA-directed *in vitro* synthesis are presented.

MATERIALS AND METHODS

Experimental procedures are described in the legends to figures and tables; only the compositions of buffers are given here. Buffer A: 10 mM Tris·acetate (pH 7.8), 14 mM Mg acetate, 60 mM K acetate, 0.1 mM dithiothreitol; TMAI: 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM 2-mercaptoethanol; TRI: 30 mM Tris·HCl (pH 7.4), 20 mM $MgCl_2$, 1 M KCl, 6 mM 2-mercaptoethanol; "Reconstitution buffer": same as TRI except 0.33 M KCl.

RESULTS

Cell-Free Protein Synthesis Directed by Escherichia coli DNA. Synthesis of several active enzymes has been demonstrated with the systems developed by Zubay (4), and Schweiger and Gold (5). In preliminary experiments we obtained better results using the former system (4, 6); we therefore decided to modify that system. Our major modification was to fractionate the "S30" of the Zubay system into salt-washed ribosomes, an initiation factor fraction ("IF"), a partially purified "enzyme fraction" ("S100DE"), and DNA-dependent RNA polymerase. S100DE contains EF-T, EF-G, and aminoacyl-tRNA synthetases, and is devoid of nucleic acids. Amino-acid incorporation in the absence of added DNA is very small (see Table 1). Further purification of these fractions has been avoided in this initial study, since the S100DE and IF fractions may possibly contain other factors necessary for r-protein gene transcription or post-translational modification of r-proteins. The amounts of each of the enzymes and factors described above giving maximum incorporation of radioactive amino acids into total protein were determined.

Requirement of the various components for incorporation of methionine into total protein is shown in Table 1. The system is dependent on presence of DNA, RNA polymerase, and ribosomes. Separation of initiation factors and other necessary enzymes into IF and S100DE fractions was apparently incomplete; omission of both fractions is necessary to abolish methionine incorporation. The strong inhibition caused by rifampicin or DNase is consistent with the conclusion that the system is largely DNA-dependent. Although about 10% of the incorporation appears to be independent of DNA, some of this residual incorporation may not reflect protein synthesis. When a ^{14}C-labeled amino-acid mixture is used in place of [^{35}S]methionine, we observe only 2–5% DNA-independent incorporation.

Isolation of Synthesized 30S Ribosomal Proteins. The *in vitro* 30S ribosome reconstitution technique (7, 8) provides an effective way to purify the newly synthesized 30S r-proteins from other protein products.

[^{35}S]Methionine was incorporated into protein with *E. coli* DNA as a template, essentially as described in Table 1. After 2 hr of incubation, the reaction mixtures were cooled and dialyzed against TRI buffer containing 8 M urea and then TMAI buffer containing 8 M urea. This treatment dissociated the ribosomes in the incubation mixture so that the released r-proteins could mix with radioactive r-proteins synthesized in the system. The resultant solution was passed through a DEAE-cellulose column (1 × 10 cm, equilibrated with 8 M urea in TMAI buffer, pH 7.4). Most of the 30S r-proteins passed through the column. However, the "acidic" r-proteins, S1, S2, and S6, were probably retained together with other "soluble proteins" and nucleic acids (9). The pass-through fraction ("basic protein fraction") contained about 50% of the total proteins.

Carrier nonradioactive 30S r-protein mixture (TP30) and 16S RNA were then added to the basic protein fraction, and 30S ribosome reconstitution was performed (7, 8). The amounts of carrier TP30 and 16S RNA giving the maximum and reproducible recovery of the radioactive r-proteins in the reconstituted 30S subunits were carefully determined (see the legends to Figs. 1 and 2). The reconstituted 30S subunits were isolated by sucrose gradient centrifugation through gradients

Abbreviations: r-protein, ribosomal protein; IF, initiation factor.

* This is paper no. 1694 of the Laboratory of Genetics.

TABLE 1. *Incorporation of [^{35}S]methionine into proteins directed by E. coli DNA*

	Relative activity (%)
Complete system	100*
− ribosomes	7.5
− IF	37
− S100DE	54
− IF, −S100DE	2.7
− RNA polymerase	7.0
− DNA	11
+ rifampicin (70 μg/ml)	10
+ DNase (70 μg/ml)	12

The reaction mixture contained the following components: "mixture" (which was identical to the "reaction mixture" as described in ref. 6 except that unlabeled methionine was omitted), 10 μl; pyruvate kinase (6.3 mg/ml), 0.5 μl; 50 mM K_3PO_4, 1 μl; [^{35}S]methionine (8 Ci/mmol),10 μl (containing 1 nmol); 3′:5′-cyclic AMP (70 mM), 1 μl; *E. coli* DNA, 20 μg in 10 μl of 10 mM Tris·acetate (pH 7.8); 70S salt-washed ribosomes (3 A_{260} units); "S100DE"; initiation factor (IF); and RNA polymerase (about 50 μg). The latter four components were prepared from *E. coli* strain PR13 (21) as described below. Together, they were dissolved in 20 μl of buffer A. The amounts of S100DE, IF, and RNA polymerase were predetermined to give maximum incorporation. Rifampicin and DNase were added as indicated. The total volume was adjusted with water to 70 μl. The reaction mixtures were incubated at 37° for 2 hr and the amount of acid-precipitable [^{35}S]protein was measured. *E. coli* strain PR13 was grown in nutrient broth containing glucose (0.8%). Exponentially growing cells were harvested and broken by alumina grinding, and extracts were made in buffer A. Ribosomes were prepared essentially as described by Crawford and Gesteland (22). IF was prepared as described before (8) except that glycerol (10%) was included in the buffer used in the DEAE–cellulose column step. S100DE was prepared from ribosome-free S100 fraction as described before (8). RNA polymerase was prepared by a method similar to the one developed by Burgess (23). The preparation was about 40% pure. DNA was prepared by a method similar to that of Marmur (24).

* 100% = 1.12 × 10^6 cpm = about 150 pmol of methionine incorporation.

made in reconstitution buffer. This buffer prevented nonspecific adsorption of nonribosomal proteins to the reconstituted particles. Fig. 1 shows the results of a typical experiment.

It can be seen that isolated reconstituted 30S subunits contained radioactive materials, presumably 30S r-proteins synthesized under the direction of the *E. coli* DNA template (Fig. 1*a*). When phage φ80 DNA was used instead of *E. coli* DNA, essentially no radioactive materials were found in the reconstituted 30S subunits (Fig. 1*b* and Table 2). Similarly, when *E. coli* DNA was omitted or DNase was included together with *E. coli* DNA, no significant amount of [^{35}S]proteins was found in the reconstituted subunits (Table 2). Together these experiments indicate that most of the radioactive proteins contained in the isolated reconstituted subunits are coded for by *E. coli* DNA, and suggest that nonspecific adsorption of radioactive proteins to the reconstituted subunits is small.

Competition experiments were performed to further test the hypothesis that the *E. coli* DNA-coded [^{35}S]proteins in the reconstituted particles were in fact 30S r-proteins. The effect

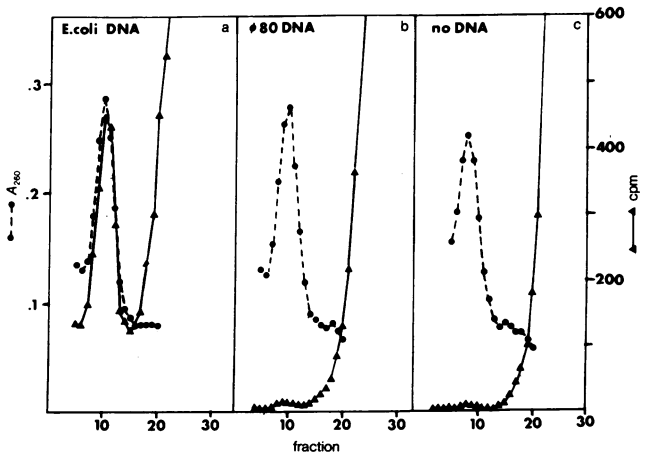

FIG. 1. Radioactive proteins synthesized *in vitro* recovered by the 30S ribosome reconstitution technique. Experiments were done as described in Tables 1 and 2, with either *E. coli* DNA (*a*) or φ80 phage DNA (*b*) as a template, or without DNA (*c*). After incubation, samples were treated as described in the text. To the basic protein fractions containing radioactive proteins, 30 A_{260} units of 16S RNA and 36 A_{260} equivalents of TP30 were added. The total volume was 4.6 ml. After reconstitution the samples were analyzed by 5–20% sucrose gradient centrifugation in a Spinco SW27 rotor for 15 hr at 22,000 rpm. Fractions were collected and aliquots from each fraction were analyzed for relative amounts of reconstituted 30S subunits (—●—) and for radioactive proteins (—▲—).

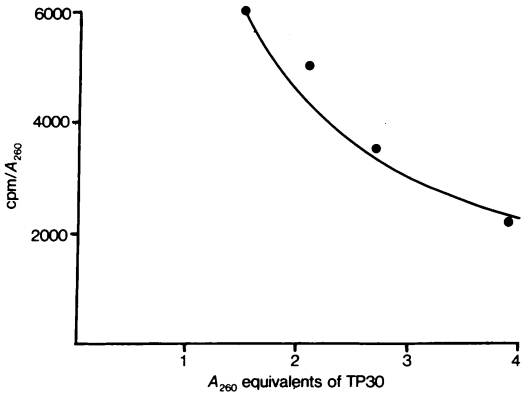

FIG. 2. Competition of excess of nonradioactive TP30 with radioactive *in vitro* synthesized proteins in 30S ribosome reconstitution. A constant amount of "basic protein fraction" containing [^{35}S]methionine-labeled *in vitro* proteins (derived from a reaction mixture containing 0.3 A_{260} units of 30S subunits) was mixed with 1.0 A_{260} unit of 16S RNA and various amounts of TP30 in a total volume of 0.27 ml. Preliminary experiments showed that the addition of 1.2 A_{260} equivalents of TP30 yielded maximum recovery of [^{35}S]proteins in reconstituted 30S subunits. Since we assume complete recovery of r-proteins derived from the 0.3 A_{260} units of 30S subunits present in the original reaction mixture, this optimum point is shown as 1.5 instead of 1.2 A_{260} equivalents of TP30 on the abscissa. Reconstitution was done at 40° and the specific activity of the reconstituted subunits was determined. The recovery of reconstituted subunits was about the same at all the TP30 concentrations. The "theoretical dilution curve" is drawn on the assumption that 1.5 A_{260} equivalents of TP30, which gave the maximum recovery of [^{35}S]proteins, is the real equivalence point.

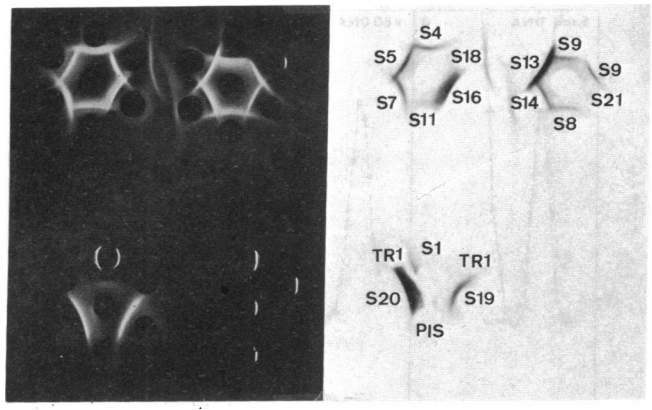

FIG. 3. Detection of proteins synthesized *in vitro* by radioimmunodiffusion. Immunodiffusion was done in 1% agar gel in TRI buffer (11). [^{35}S] proteins extracted from the reconstituted 30S subunits by an RNase digestion method (8) were placed in each center well of the three sets. The outer wells received antisera against individual r-proteins as indicated. Pooled preimmune serum (*PIS*) was added as a control. After 3 days at room temperature, the plate was photographed (*left*). The gels were washed extensively by repeated soaking in TRI buffer and finally in water. The gels were covered with a piece of filter paper, dried, and exposed to x-ray film. The autoradiogram obtained is shown (*right*).

of adding excess nonradioactive carrier TP30 to a constant amount of [^{35}S]proteins and 16S RNA on the incorporation of 30S [^{35}S]r-proteins into reconstituted 30S subunits was measured. The results presented in Fig. 2 show that the specific activity of the reconstituted 30S subunits was decreased by the dilution of a constant amount of the radioactive putative 30S r-proteins with excess amounts of nonradioactive 30S r-proteins. We conclude that most of the radioactive proteins synthesized under the direction of *E. coli* DNA and recovered by the above reconstitution technique are very similar or identical to genuine 30S proteins.

Identification of Synthesized 30S r-Proteins. Radioactive proteins recovered in the reconstituted 30S subunits were examined by a radio-immunodiffusion technique using antisera prepared against various pure *E. coli* 30S proteins (10, 11). The presence of proteins S2, S3, S6, S12, S15, and S17 was not tested. The following radioactive 30S proteins were detected by autoradiography of immunodiffusion plates: S4, S5, S7, S8, S9, S10, S13, S14, S16, S19, and S20 (Fig. 3 and other experiments not shown). Proteins S1, S11, S18, and S21 were not detected by this method. Since proteins S18 and S21 synthesized *in vivo* lack methionine (10, 12), we would not detect proteins synthesized *in vitro* that were identical to mature S18 or S21. However, *in vitro* synthesized S18 has been detected among the reaction products before the reconstitution step when [^{14}C]amino acids were used in place of [^{35}S]methionine (unpublished results). These observations suggest that either precursor S18 containing formylmethionine is not incorporated into reconstituted 30S particles, or that such precursor proteins are rapidly matured in our system, or that the *in vitro* synthesis of S18 does not involve a formylmethionine-containing precursor. Negative results were also expected for S1, since it would have been removed by both the DEAE–cellulose and reconstitution steps (9, 13). Since the [^{35}S]proteins contained in the samples are mainly 30S r-proteins (see the previous section) and since the negative results obtained

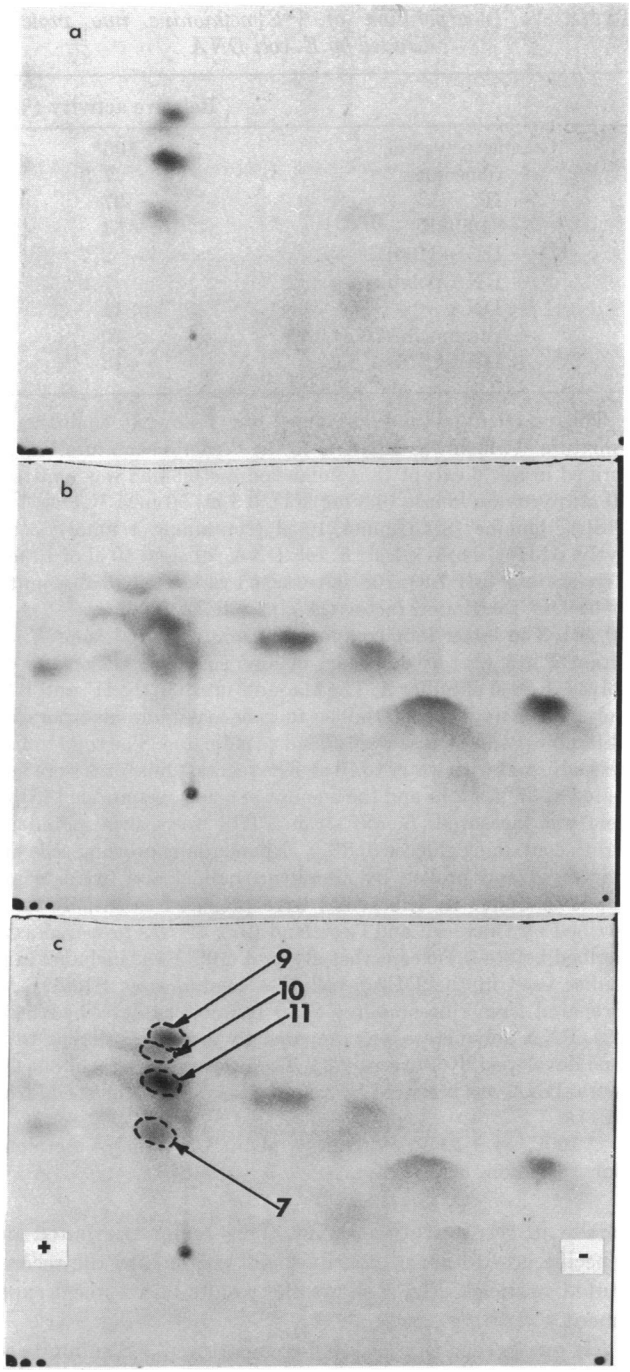

FIG. 4. Tryptic peptide map of [^{35}S]methionine-labeled S13 synthesized *in vitro*. (*a*) Autoradiogram, (*b*) ninhydrin-stained map, and (*c*) the autoradiogram superimposed on the ninhydrin-stained map. Separation of peptides was done first by electrophoresis and then by chromatography as described previously (10).

with S1, S18, and S21 indicate lack of nonspecific adsorption of contaminating proteins to immunoprecipitin bands in this technique, we believe that positive identification of radioactive proteins by the present technique can be trusted.

More rigorous proof of synthesis of r-proteins was obtained for protein S13, which showed a strong band in the autoradiogram of the radio-immunodiffusion experiments. [^{35}S]Methionine-labeled 30S r-proteins synthesized *in vitro* were isolated by the reconstitution technique as described above, reex-

tracted from the ribosomes, and S13 was precipitated with an equivalent amount of anti-S13 serum. The precipitate was washed, dissolved with 8 M urea–4 M LiCl, mixed with nonradioactive pure S13, and digested with trypsin. The tryptic peptides were separated by a two-dimensional fingerprinting technique on silica gel thin-layer plates. The separated peptides were stained with ninhydrin and the radioactive peptides were detected by autoradiography. Because of the large excess of added carrier S13, relative to that of the immune precipitate, these stained peptides represented mostly the peptides from S13.

As shown in Fig. 4, two strongly radioactive peptides were coincident with the peptides 9 and 11 detected by ninhydrin staining. Two weakly radioactive peptide spots were found, one of which was coincident with peptide 7, detected by ninhydrin staining; the other (called peptide 10) did not have any corresponding spot detected by ninhydrin. All the stained peptides, as well as the material from the position corresponding to peptide 10, were then analyzed for their amino-acid compositions (for technique, see ref. 10). Only two peptides, 9 and 11, were found to have methionine in amounts close to 1 mol/mol of peptide. Peptide 7 showed only a small amount of methionine relative to other amino acids. No methionine was detected in any of the remaining peptides analyzed, including peptide 10.

Since the two major radioactive peptides were exactly coincident with the two methionine-containing peptides 9 and 11 (see Fig. 4*c*), the results strongly support the conclusion that a protein very similar or identical to S13 in its primary amino-acid sequence was synthesized in the present cell-free system. Protein S13 has only two methionine residues per molecule (12). The origin of small amounts of radioactive material in peptide 7 and spot 10 has not been determined.

Similar tryptic peptide analysis was used to identify several other r-proteins. In this case, radioactive putative 30S r-proteins isolated by the reconstitution technique were separated by one-dimensional polyacrylamide-gel electrophoresis in urea at pH 4.5 (8, 14). After staining, the proteins were extracted from gel slices and digested with trypsin. The same procedure was carried out with *E. coli* 30S r-proteins labeled *in vivo* with [^{35}S]methionine. Tryptic peptides from [^{35}S]proteins synthesized *in vitro* and those from the corresponding [^{35}S]proteins made *in vivo* were subjected to electrophoretic separation on paper followed by autoradiography, and the radioactive peptides were compared.

Fig. 5 shows some examples of the results. One-dimensional polyacrylamide-gel electrophoresis did not completely separate all the 30S proteins. Proteins extracted from isolated bands such as S10 (Fig. 5*a*) and S20 (Fig. 5*b*) showed simple peptide patterns, and proteins labeled both *in vitro* and *in vivo* gave identical or very similar peptide patterns (Fig. 5*a* and *b*). Proteins extracted from bands that either contained several proteins or were very close to neighbor bands showed complex patterns of peptides. For this reason, rigorous proof of the identity of peptides from *in vitro* products from these bands was not possible. An example is shown in Fig. 5*c*, in which peptides were obtained from the band containing S12, S13, S15, S16, and S17.

Such rigorous peptide analysis was done only for S10 and S20. However, most of the radioactive proteins synthesized *in vitro* had mobilities in polyacrylamide-gel electrophoresis similar to those of the reference r-proteins. These observations, together with the results given in Fig. 2, strongly suggest that at least a major part of the *in vitro* synthesized proteins isolated by the reconstitution technique consists of proteins very similar or identical to genuine 30S r-proteins.

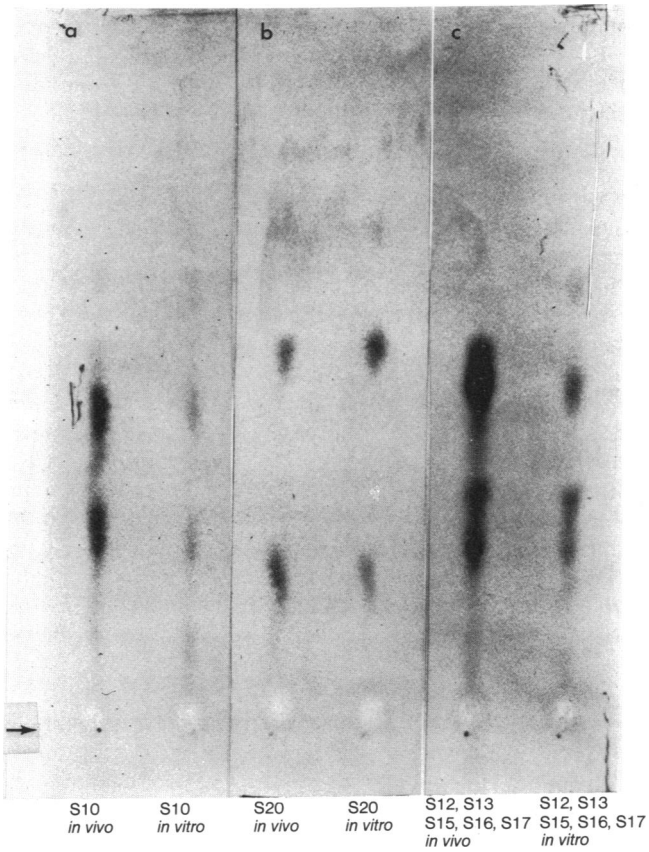

FIG. 5. Autoradiograms of ^{35}S-labeled tryptic peptides obtained from S10, S20, and a protein mixture eluted from a band on a polyacrylamide gel column. Concerning the protein mixture in the third set, (*c*), see the *text*. ^{35}S-labeled peptides were separated by electrophoresis on Whatman 3MM paper in a buffer containing 10% acetic acid, 1.37% pyridine, pH 3.6, at 2100 V for 2.5 hr. Autoradiograms of the papers were then made. *Arrow* indicates the origin.

Table 2 gives quantitative data on the amounts of 30S r-proteins synthesized under several conditions. Here the

TABLE 2. *Incorporation of [^{35}S]methionine into total and ribosomal proteins directed by E. coli and φ80 phage DNAs*

DNA and other additions	Incorporation into: Total proteins (10^6 cpm)	Incorporation into: 30S r-protein fraction (10^4 cpm)	Percent 30S r-proteins
E. coli DNA	38	13.7	0.36
φ80 DNA	8.5	0.25	0.025
E. coli DNA + DNase	4.0	0.19	0.048
No DNA	4.3	0.07	0.016

Experimental conditions were similar to those described in Table 1. Final concentration of DNAs: *E. coli* DNA, 300 μg/ml; φ80 DNA, 80 μg/ml. The values listed as "incorporation into 30S r-protein fraction" are equal to the total radioactivity recovered in the purified reconstituted 30S subunits multiplied by an appropriate correction factor (usually about 2) based on the partial recovery of added 16S RNA in the purified subunits.

assumption is made that all of the radioactive proteins isolated by the reconstitution technique represents 30S r-proteins. The table shows that about 0.4% of total proteins synthesized in the presence of *E. coli* DNA are 30S r-proteins.

DISCUSSION AND CONCLUSIONS

DNA-directed *in vitro* synthesis of 30S r-proteins has been demonstrated. Although such synthesis has been rigorously demonstrated only with respect to S10, S13, and S20, the data presented above strongly indicate that at least eight other 30S r-proteins are synthesized in the present system. We have not studied synthesis of 50S r-proteins in this system.

The calculation given in Table 2 shows that only about 0.4% of the proteins synthesized in this system are 30S r-proteins. About 3–10% of total proteins synthesized in growing *E. coli* cells are 30S r-proteins (1), demonstrating a clear discrepancy between the present *in vitro* system and the intact cell. Although part of this difference may reflect errors in the estimation of the amount of r-protein synthesized *in vitro*, several other explanations are possible. For example, the *in vitro* system might be deficient in some important factors necessary for the transcription of r-protein genes or for the translation of r-protein mRNAs, or the primary translational products of r-protein mRNAs might differ from the mature r-proteins and might not be incorporated into 30S subunits by the reconstitution step.

The present system may be a useful assay for factors which might influence the expression of r-protein genes. In addition, possible r-protein precursors might be studied in the present system. Some experiments along these lines have already been initiated. Preliminary experiments have shown that the omission of 2′:3′-cyclic AMP does not significantly affect synthesis of r-proteins. Hence, the expression of r-protein genes appears to be different from that of catabolite-sensitive genes, such as those in the *lac* operon (4, 15). The effect of guanosine tetraphosphate, ppGpp, which is believed to be involved in the regulation of rRNA gene expression (16, 17), was also studied. Weak inhibition of r-protein synthesis was observed at concentrations between 0.1 and 1 mM. The biological significance of this observation is not clear.

The synthesis of r-proteins in this system is dependent upon the presence of the proper DNA, *E. coli* DNA, in the present study. In addition to using ϕ80 phage DNA as a template, as shown in Table 2, we have also tested the activity of DNA from a defective ϕ80-transducing phage which carries cistrons both for 16S and 23S rRNA (a gift from Dr. L. Soll, see ref. 18). No 30S r-protein synthesis was detected. The DNA from this transducing phage has been shown to be a good template for the synthesis of rRNA (R. Almendinger and M. Nomura, unpublished experiments). Thus absence of r-protein synthesis with this DNA as a template appears to exclude the hypothesis (19, 20) that "nascent" rRNAs are the templates for r-proteins. However, it is still possible that nascent rRNAs have some role in the synthesis of r-proteins. It is hoped that the present system will also be useful for the study of mechanisms of possible coordinated expression of r-protein genes and rRNA genes in growing bacterial cells.

We thank C. Nelson for her technical help, Dr. R. R. Burgess for his advice on preparation of RNA polymerase, and Dr. J. E. Dahlberg for his reading of the manuscript. This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-20427) and the National Science Foundation (GB-31086).

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