THE MESSENGER-DIRECTED SYNTHESIS OF THE α-FRAGMENT OF THE ENZYME β-GALACTOSIDASE

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Received June 4, 1973

SUMMARY: A system has been developed which translates lac messenger RNA (mRNA) from Escherichia coli in vitro. This system synthesizes the N-terminal portion of the β-galactosidase molecule (the α-region). The use of a complementation system allows enzymatic assay of this de novo synthesized material. Synthesis of this fragment is blocked by translational but not transcriptional inhibitors, as expected for an mRNA-directed system. The lac mRNA which can direct α-fragment synthesis has a heterogeneous size distribution over the approximate range 0.4 - 1.8 x 10^6 daltons. The system is not affected by adenosine 3',5'-cyclic monophosphate (cAMP) nor isopropyl-β-D-thiogalactopyranoside (IPTG), but is inhibited by the guanine nucleotides ppGpp and pppGpp.

Messenger RNA-directed systems for the in vitro synthesis of specific proteins provide an important tool for the study of translational processes. Synthesis of a specific protein provides the most sensitive and direct means for detecting the presence of a biologically competent mRNA. Moreover, such factors as chemical modification of the mRNA and variation of ionic strength or divalent cation concentration can be evaluated in these systems for their influence on efficiency of translation. In addition, gene controlling elements, such as repressors or positive control factors, can be examined to determine whether control is exerted wholly or partly at the post-transcriptional level.

Several cell-free systems have been developed for translation of eucaryotic (1) and bacteriophage mRNAs (2). There have also been recent reports of successful translation of bacterial mRNAs in vitro (3, 4). In the latter reports too little protein was synthesized to be detectable enzymatically; because of this, indirect characterization of the in vitro product was made. This entails copurification of radioactively labelled protein, made in vitro, with bona fide cold carrier enzyme. Such a technique is more time consuming than characterization by direct enzymatic assay, as well as being subject to the possible pitfall of nonspecific interaction between carrier and labelled material.

Here we report the cell-free synthesis of the α-peptide of β-galactosidase (β-gal) under the direction of lac mRNA. This fragment can be assayed enzymatically using the α-complementation system developed by Ullman, Jacob and Monod (5). Some features of this system and of the mRNA which directs the synthesis are described.

MATERIALS AND METHODS: E. coli strain Z191^{q}, which carries a mutant lac repressor and β-gal, is used to prepare bacterial extracts and has been described elsewhere (6). E. coli strain CA 159, used as a source of lac mRNA, is constitutive for expression of the lac operon. It was obtained from J. Beckwith.

The procedures for cell growth and preparation of the S-30 bacterial extract have been described (7). Components of the incubation mixture were as previously reported (7) with the following exceptions: cAMP was omitted; 16 mg/ml of polyethylene glycol (Carbowax 6000, Union Carbide Corporation) were present; concentrations of Mg^{++} and Ca^{++} were 16mM and 8mM, respectively.

Crude lac mRNA was made as follows. Seventy gms of freshly grown cells of strain CA 159 were chilled, combined with two volumes of H_2O and 1.7 volumes of 88% phenol, and shaken vigorously for 1 hour at room temperature. The mixture was centrifuged at 16,000 x g for 30 minutes at 4°. The aqueous phase was carefully decanted and subjected to 2 additional extractions with equal volumes of 88% phenol. Subsequent manipulations were performed at 4°. The final aqueous layer was combined with 2 volumes of cold absolute ethanol, allowed to stand for 10 minutes, then centrifuged at 27,000 x g for 5 minutes. The precipitate was resuspended with stirring in 60 ml of 2M NaCl, then centrifuged at 27,000 x g for 30 minutes. The supernatant was discarded and the pellet was suspended in 30 ml of H_2O and dialyzed overnight against H_2O. Undissolved material was removed by centrifugation for 1 hour at 27,000 x g and the clear supernatant was frozen in 2-3 ml aliquots and stored at -20°. The frozen material was completely stable for at least 2 months. This fraction, which was used as the source of RNA to direct protein synthesis, consists primarily of ribosomal RNA (rRNA) and mRNA and is relatively free of DNA, transfer RNA and protein.

Incubations and assays were performed as described (7) except that RNA, rather than DNA, was used to direct enzyme synthesis and incubations were carried out for 10 minutes at 36°.

Bovine pancreatic ribonuclease 11-A was obtained from Sigma Chemical Company and RNase-free DNase from Worthington Biochemical Corporation.

RESULTS AND DISCUSSION: We have adapted the DNA-directed in vitro system originally developed in our laboratory for use in RNA-directed protein synthesis. This system consists of an S-30 (which is a crude, cell-free extract preincubated to remove endogenous mRNA), substrates, cofactors and inorganic ions necessary for protein synthesis, and added RNA. These are mixed, incubated for an appropriate time and then assayed for enzymatic activity.

Ullman et al. (5) have found that strains of E. coli which carry a deletion in the operator proximal 20-25% of the z gene of the lac operon (the

TABLE I

Effect of Various Agents on RNA-Directed *In Vitro* Protein Synthesis

INCUBATION SYSTEM	RELATIVE β-GAL YIELD
Complete System	100
Zero Time of Incubation	0
No RNA	1
RNase 5 µg/ml	0
DNase 5 µg/ml	90
Puromycin 100 µg/ml	1
Chloramphenicol 100 µg/ml	5
Rifampicin 2 µg/ml	122
Actinomycin D 2 µg/ml	116
S-30 from strain LG 4 instead of Z191^q	3

Incubations were performed as described in Materials and Methods using an S-30 from strain Z191^q and 2800 µg RNA/ml. Aliquots were removed from the incubation mixture after 10 minutes at 36° and assayed for β-galactosidase. Results shown are the averages of duplicate experiments. Duplicates generally varied by less than 10%. After a 24-hour assay, the A_{420} of the complete system was about 1.0.

α-region) produce a protein which lacks β-gal activity. If enzymatically inactive α-peptides are mixed with this protein, some enzymatic activity results from complementation. We have made use of this complementation system by preparing our S-30 from strain Z191^q which produces the enzymatically inactive α-receptor protein. Thus, the α-fragment which is synthesized *de novo* in the cell-free system will complement the protein present in the S-30 and give β-gal activity. In this way RNA-directed protein synthesis can be assayed enzymatically. This procedure has been used in this laboratory for detecting DNA-directed protein synthesis as well (8).

The results in Table I demonstrate RNA-directed *de novo* synthesis of a polypeptide containing the α-fragment. The amount of enzyme produced is un-

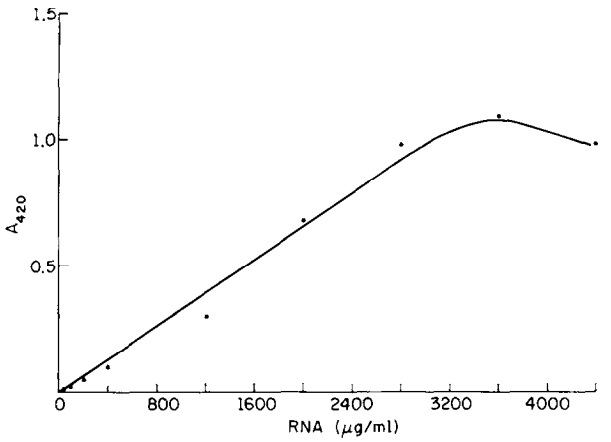


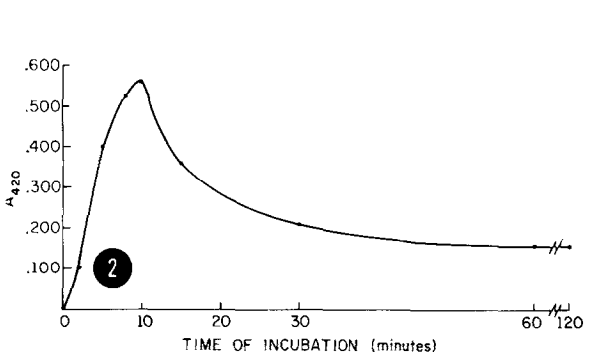
Figure 1. β-Galactosidase Activity as a Function of RNA Concentration.

Experiments performed as described in the legend to Table 1, except for varying RNA concentration. Assay time was 24 hours.

affected by DNase and the transcriptional inhibitors rifampicin and actinomycin D. On the other hand, RNase and the translational inhibitors puromycin and chloramphenicol prevent or block synthesis. When strain LG 4, with a deletion of the entire β-gal gene, is used to prepare the S-30, little enzymatic activity is detectable. This indicates that cell-free synthesis of the entire β-gal polypeptide is not possible. A similar result has been obtained for mRNA-directed cell-free synthesis of tryptophanase by Parish *et al.* (4).

Figure 1 shows that enzymatic activity is a linear function of total RNA added to the system over the range 50 - 3000 μg/ml. It seems likely that *lac* mRNA comprises a small fraction of this RNA (0.1 - 0.2%) but no reliable estimate is yet available. The broad Mg^{++} optimum at 2800 μg RNA/ml peaks at 16mM (data not shown). The ionic strength optimum under these conditions is about 0.02M higher than that used for DNA-directed synthesis.

Figure 2 shows enzyme production as a function of synthesis time. The system produces a maximum amount of enzyme when a synthesis time of about 10 minutes is used. The early maximum is consistent with the known instability of *lac* mRNA *in vivo* (9, 10). In contrast a DNA-directed system produces β-gal at a linear rate for at least 2 hours under the conditions used here. When



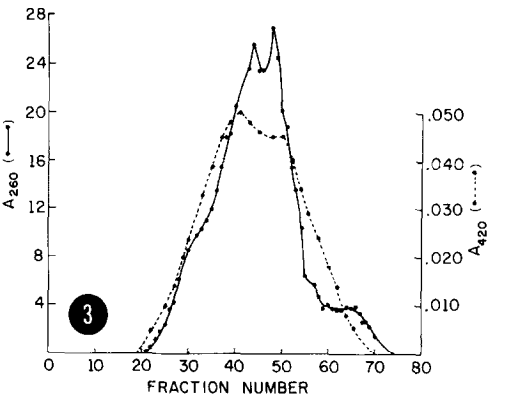


Figure 2. β-Galactosidase Activity as a Function of Time of Incubation.

Experiments performed as described in the legend to Table I, except that aliquots were removed from the incubation mixture for assay at the times indicated. Assay time was 16 hours.

Figure 3. Sepharose 4B Column Profile.

Approximately 700 A_{260} units of crude RNA in 2.5 ml of 0.05M Tris-acetate, pH 8.2, and 0.1 mM EDTA were applied to a 1.6 x 60 cm column of Sepharose 4B preequilibrated with the above buffer at 4°. Using this buffer, the column was run at a flow rate of 8ml/hr and 1.35 ml fractions were collected. Fractions were assayed for A_{260} and 0.11 ml aliquots were assayed, without further treatment, for β-gal synthesizing activity as described in the legend to Table I. Assay time for β-gal was 30 hours. Recovery of A_{260} units was 95%, of β-gal synthesizing activity, 70%.

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the synthesis time is longer than 10 minutes, less enzyme is detected; this net loss of enzymatic activity suggests that the complemented protein, as well as the mRNA, is unstable. It is not unreasonable that the active enzyme in our system, consisting of two peptide fragments associated in vitro, is more susceptible to protease attack than a single polypeptide chain would be. Consistent with this notion is the observation that addition of a serine protease inhibitor, p-toluene sulfonyl fluoride, to the system increases maximum enzyme yields about 25% (data not shown).

The crude RNA preparation was applied to a column of Sepharose 4B in order to examine the size distribution of the mRNA which can direct α-fragment synthesis (Fig. 3). The main peak of mRNA runs ahead of the rRNA. This is

TABLE II

Effect of Small Regulatory Molecules on RNA-Directed *In Vitro* Protein Synthesis

INCUBATION SYSTEM	RELATIVE β-GAL YIELD
Complete System	100
5×10^{-4}M IPTG	97
5×10^{-4}M cAMP	101
5×10^{-4}M IPTG and 5×10^{-4}M cAMP	96
1.2×10^{-4}M ppGpp	81
3.0×10^{-4}M ppGpp	66
1.2×10^{-4}M pppGpp	87
3.0×10^{-4}M pppGpp	66

Experiments performed as described in legend to Table I.

consistent with its being intact *lac* mRNA, having an expected size of 1.8×10^6 daltons, based on the molecular weights of the proteins encoded (11, 12). In addition there is a prominent shoulder of mRNA running somewhat more slowly than rRNA. This mRNA, approximately 4×10^5 daltons, is too small to code for the entire β-gal molecule of 135,000 daltons. Because it synthesizes the N-terminus of β-gal which contains α, however, it must contain (very nearly) the 5' terminus and lack the normal 3' terminus of *lac* mRNA. Thus it may be a correlate from living cells of the mRNA fragment obtained by DeCrombrugghe *et al.* (13) in a purified transcriptional system in the presence of excess ρ termination factor. At present, however, it is possible that this species arises as an artifact during preparation of the RNA. The broad distribution of RNA capable of synthesizing the α-fragment of β-gal may indicate considerable size heterogeneity in the *lac* mRNA population, as has been suggested by others on the basis of hybridization experiments (14).

The *lac* operon *in vivo* is subject to both a negative control system, effected by *lac* repressor and an inducer such as IPTG, and a positive control

system effected by catabolite gene activator protein and cAMP (15). Both systems are believed to operate at the transcriptional level (16). To test this conclusion, we examined the effects of IPTG and cAMP on the mRNA-directed system. As seen in Table II, neither compound has an effect, singly or in combination, at levels where DNA-directed protein synthesis is greatly stimulated (7). This strengthens the belief that these factors operate exclusively at the transcriptional level.

The stringent phenomenon of cessation of rRNA synthesis in response to amino acid starvation in E. coli is controlled by the rel locus (17). The product of this locus has recently been shown to stimulate the synthesis of the guanine nucleotides ppGpp and pppGpp (18). In a DNA-directed in vitro protein synthesizing system, these compounds, at 10^{-4} M, stimulate β-gal production about two fold (19). In contrast, both compounds slightly inhibit α-fragment synthesis in the mRNA-directed system (Table II). This may indicate the presence of translational control of protein synthesis as a component of the overall stringent control phenomenon. Nonspecific inhibition of the system by these nucleotides cannot be excluded, however.

This mRNA-directed protein synthesizing system, in conjunction with the DNA-directed coupled system previously developed promises to be a useful tool for discrimination between factors affecting transcriptional versus translational components of bacterial gene regulatory processes.

ACKNOWLEDGEMENTS: We are grateful to Tetteh Blankson for technical assistance. One of us (G. R.) was a recipient of a predoctoral fellowship from the National Institutes of Health (1 F01 GM 46917). This work was supported by a grant from the National Science Foundation (NSF GB 18733). The ppGpp and pppGpp were obtained from M. Cashel.

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