**A novel in vitro transcription-translation system: accurate and efficient synthesis of single proteins from cloned DNA sequences**

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A system is described which permits the efficient synthesis of single proteins in vitro. The essential element in this expression system is a strong promoter derived from coliphage T5 which produces, with high efficiency, specific RNAs in capped or uncapped form, depending upon the experimental conditions used. The transcription-coupled capping of RNA allows the direct translation of the RNA in eukaryotic extracts from wheat germ as well as from HeLa cells. The synthesis of three different proteins is reported, including lysozyme, which is shown to be translocated across membranes when appropriate assay conditions are used. The simplicity of the experimental procedure, the high purity and specific activity of the [35S]methionine-labelled proteins produced offer a number of possibilities for the study of structure-function relationships of proteins.

Key words: transcription-coupled translation/T5 promoter/membrane translocation

**Introduction**

Cell-free protein synthesizing systems have been instrumental in studying mechanisms of protein biosynthesis. They have also proved to be important tools for the analysis of membrane biogenesis and the translocation of proteins across, or integration into membranes (Blobel and Dobberstein, 1975; see Sabatini et al., 1982). A prerequisite in many of these studies is the availability of individual well defined mRNA species. There are, however, only a few cases in which suitable mRNA species can easily be purified from specialized tissues or cell lines.

With the advent of efficient cDNA cloning and screening methods, it became feasible in principle to produce defined transcripts of any sequence encoding a protein of interest. Furthermore, proteins can be produced from modified DNA and subsequently tested in biological assays. Such an approach has already provided valuable information on structural features of signal sequences (Silhavy et al., 1977; Bedouelle et al., 1980). What is now required is a simple efficient system in which selected DNA segments can be transcribed into functional mRNA and these then translated into proteins. An 'ideal' in vitro expression system would express a single protein species from cloned DNA in both prokaryotic and eukaryotic lysates in a quick and simple procedure.

Here we describe a method which permits the production of single proteins in coupled transcription-translation systems of pro- and eukaryotic origin (Roberts et al., 1975; Yang et al., 1980; Paterson and Rosenberg, 1979). The salient feature of the system is the highly selective and efficient synthesis of mRNA using a promoter of coliphage T5 (Bujard, 1980) which, depending upon the experimental conditions, produces capped or uncapped mRNA in high yields.

**Results**

**The experimental design**

The most important step in the exclusive expression of a single protein species in vitro is the selective transcription of the gene of interest. This is achieved by cloning the proper sequence into the plasmid PDS6 whose main properties are as follows (see also Figure 1). (i) It contains multiple insertion sites between the strong coliphage promoter P25X/O and the lambda terminator t5 (Figure 2). (ii) Downstream of t5 is located the promoter-free gene of chloramphenicol acetyltransferase (cat), which carries its own ribosomal binding site (RBS) and which is followed by a second terminator. (iii) The replication region (ori) and the beta-lactamase gene (bla) are pBR322 derived. (iv) Using the proper in vitro transcription conditions, P25X/O outcompetes the beta-lactamase promoter 20-fold, thus, ~95% of potentially translatable RNA is specified by the phage promoter. (v) There is no ATG between the transcriptional start sequence and the most distal site for gene insertion. (vi) Under conditions described in Materials and methods P25X/O efficiently initiates transcripts with 7mGpppA resulting in capped mRNAs.

A foreign gene integrated downstream of the phage promoter can be expressed in vitro systems with high efficiency using Escherichia coli RNA polymerase for transcription and purified pro- or eukaryotic cell lysates for translation. Supplying [35S]methione, the protein of interest can be obtained as labelled product of high specific activity, tested for different biological functions (e.g., translation into membranes) and then be directly subjected to biochemical analyses (e.g., electrophoresis followed by autoradiography).

Since in vitro as well as in vivo 3–5% of transcriptional readthrough occurs at terminator t5 some bicistronic mRNA will exist in both situations. In vivo this results in a moderate but distinct chloramphenicol resistance of the cell which can be utilized for controlling the integrity of the transcriptional unit constructed.

**Structure and properties of the plasmid systems pDS5 and pDS6**

All plasmids described here are derived from the plasmid family pDS1 (Stueber and Bujard, 1982; Bujard et al., 1983). These vectors were designed for the cloning and quantitative analysis of prokaryotic transcription and translation signals. The pDS plasmids contain the bla gene, with its original promoter (P3, Stueber and Bujard, 1981) as an internal transcriptional standard. For analysing various expression signals, these plasmids carry two indicator genes, dhfr (dihydrofolate reductase) and cat which can be put under the
control of a single promoter. There are several advantages to using these indicator genes: (i) their products are tolerated by the E. coli cell in large amounts and are easily assayable; (ii) the respective sizes of their genes are only 665 and 745 bp; thus, the plasmids are still relatively small, which facilitates the introduction of additional unique cleavage sites as well as the insertion of further genetic material. Other advantages of the pDS systems relating to their structural stability, their maintenance in E. coli and their potential as efficient expression systems have been described previously (Bujard et al., 1983, 1984).

The most relevant properties of the plasmids pDS5 and pDS6 are described in Figure 2. They all have in common the region spanning from the Xbal to XhoI cleavage sites, which corresponds to the PvuII/EcoRI fragment of pBR322. All constructs contain the promoter/operator fusion P_{N25X/O} and the cat gene carrying its original ribosomal binding site. The main differences of the various constructs concern the region between the promoter and the cat gene and the integration of transcriptional terminators upstream and downstream of the cat region. Thus, in pDS5/3 and pDS5/2 the dhfr sequence is present with and without a ribosomal binding site, respectively. This allows the in vivo production of DHFR in the case of pDS5/3, whereas only CAT is synthesised with pDS5/2 (Figure 3A). The absence of visible amounts of CAT in cells carrying pDS5/3 is due to the terminator t_{3} between the dhfr and the cat gene. Elimination of the dhfr sequence from pDS5/2 results in pDS5/1 which in turn was used for the construction of pDS5 and pDS6. Both of these plasmids contain a polylinker with six unique cleavage sites for the convenient integration of foreign genes and the terminator t_{1} of the rmmB operon (Brosius et al., 1981) downstream of the cat region. The only difference between the two plasmids is the presence of t_{5} in pDS6 to reduce transcriptional readthrough into the cat region. Integration of the lysozyme gene leads to pDS5/4 which has been used in the experiments described below.

Translation of in vitro transcribed mRNA from pDS5/1–3 in the wheat germ cell-free system

Translation of a mRNA by E. coli ribosomes is dependent on a functional ribosome binding site. In contrast, eukaryotic ribosomes are thought to recognize the 5’ end of the mRNA, to move along the mRNA and to initiate protein synthesis at the first AUG (Kozak, 1983). For an efficient recognition of a mRNA by eukaryotic ribosomes, the 5’ end of the molecule has to be ‘capped’ by 7-methylguanosine.

Capping coupled to transcription was achieved in vitro by including 7mGpppA in the transcription assay (Contreras et al., 1982). Unlike 7mG which is a potent inhibitor of polypeptide chain initiation, 7mGpppA has no inhibitory effect at the concentrations used (data not shown).

Using coliphage T5 promoter P_{N25X/O} as the transcriptional start signal in plasmids pDS5/1–3, capped mRNAs were obtained in the presence of 7mGpppA and E. coli RNA polymerase in good yields. Since these mRNAs are by far the most abundant translatable species in the assay, virtually single proteins are produced in each case using a wheat germ cell-free translation system. In Figure 3B the individual proteins are shown: DHFR (from pDS5/2), an extended form of DHFR (from pDS5/3) and CAT (from pDS5/1). As expected in eukaryotic systems, the translation was always initiated at the first AUG codon of the corresponding mRNAs. The difference between pro- and eukaryotic translation initiation is demonstrated best with pDS5/2: whereas in bacteria only CAT is synthesised from the bicistronic mRNA (there is no ribosomal binding site for DHFR), the eukaryotic in vitro translation system gives rise to only DHFR – provided 7mGpppA is used in transcription (Figure 3A,B).

Translation in the HeLa cell free system

To test whether the in vitro produced capped mRNA can also be translated in a mammalian cell-free system, mRNA derived from pDS5/2 was translated in a HeLa cell extract. As seen in Figure 4, lane 3, translation of DHFR-mRNA was
achieved with an efficiency comparable with that obtained when an optimal concentration of mRNA from myeloma tumor MOPC 41 was translated under identical conditions.

Fig. 2. Schematic description of plasmids. (A) Genetic and physical map of pDS5 and pDS6. Both plasmids contain the region between PstI (now XbaI) and EcoRI (now XhoI) of pBR322 carrying the replication region (ori) and the bla gene. The polylinker from pUC8 (Vienna and Messing, 1982) is placed between Pm2-S-O (a fusion of coliphage T5 promoter Pm2 with the lac operator of E. coli, to be published elsewhere) and the promoter-free gene for CAT which still contains its original RBS. Cleavage sites interfering with the utilization of the polylinker were removed from the plasmids. Transcripts from P are terminated in vivo and in vitro at terminator tR of the rrmB operon of E. coli and in addition also at terminator tL of phage λ in the pDS6 system. The lower part of the figure shows the sequence between the -43 region of Pm2-S-O of pDS5 and the start codon of the cat gene as well as the insertion site of the terminator tR of phage λ as it has been used to convert pDS5 to pDS6. (B) Schematic description of plasmids pDS5/1-4. These plasmids differ from pDS5 within the region between the E. coli and HindIII site. pDS5/1: the 36-bp fragment of pDS5 is shortened to a 13-bp EcoRI-HindIII fragment. pDS5/2: the coding region of the dhfr gene (187 amino acids) is inserted. pDS5/3: a synthetic ribosomal binding site (RBS) is inserted in front of the dhfr gene resulting in a 192 amino acid fusion protein; transcripts are terminated in vivo at terminator tL, pDS5/4: the CDNA for chicken lysozyme (lsn) has been inserted into the HindIII site of pDS5/1. For convenience, ATGs from which protein synthesis is initiated are underlined and relevant restriction cleavage sites are denoted. In contrast to pDS5 and pDS6 in pDS5/1-4 the PstI and HindIII sites in the bla gene and the EcoRI site in the cat gene are still present. Furthermore, plasmids pDS5/1, 2 and 4 contain terminator tR at the XbaI site (replacing tL), whereas in pDS5/3 this termination signal is located between dhfr and cat.

MOPC 41 mRNA primarily directs the synthesis of light chains of IgG (Blobel and Dobberstein, 1975) (Figure 4, lane 2).

Translation and membrane translocation of chicken lysozyme

To study functional features of signal sequences involved in the translocation of proteins across membranes we cloned the chicken lysozyme cDNA into the HindIII site of pDS5/1. After transcription with E. coli RNA polymerase and translocation of the capped mRNA in the wheat germ cell-free system, a single protein was obtained with the mol. wt. of prelysozyme (Figure 5, lane 1). To test whether this protein can be translocated across the membrane of the endoplasmic reticulum, translation was carried out in the presence of either Signal Recognition Particle (SRP), or salt-washed rough microsomes (RM40p) or of both SRP and RM40p.
translocated into microsomal vesicles in an SRP-dependent manner, typical for secretory proteins. As expected SRP inhibits the production of prelysozyme and RM_k by itself can neither translocate nor process prelysozyme. Mature lysozyme is found inside the microsomal vesicles only if SRP and RM_k are both present during translation, as judged by the protection of mature lysozyme against exogenous proteinase K. This protection is eliminated upon solubilization of the membrane by Triton X-100.

Discussion

The experimental system described here permits the efficient expression of protein-coding DNA sequences in vitro. The pertinent element in this system is a strong prokaryotic promoter derived from coliphage T5 which accepts ATP as well as 7mGpppA for initiation of transcription. Consequently, depending upon the experimental conditions, capped or uncapped mRNAs can be produced in a simple transcription assay using E. coli RNA polymerase. Several promoters of phage T5 have been found to belong to the strongest transcription initiation signals of the E. coli system. In vitro and in vivo

1 2 3 4 5 6 7

Fig. 4. Protein synthesis in the HeLa cell-free system. The translation system was supplemented with no RNA (lane 1), 10 µg/ml mRNA from MOPC 41 myeloma tumour (which produces mainly mRNA for the light chain of IgG, pLi) (lane 2) and an aliquot of an in vitro transcription assay with plasmid pDSS/2 as template (lane 3). The second major protein synthesised under these conditions must be related to DHFR as it can be precipitated with anti DHFR antibodies. The assays were processed and evaluated as described in the legend to Figure 3B. The HeLa cell-free system was as described before (Garoff et al., 1978).

they outcompete any other promoter for RNA polymerase if proper conditions are provided (Gabain and Bujard, 1979; Gentz et al., 1981; Deuschle and Bujard, in preparation; Gentz and Bujard, in preparation). Therefore, in the expression system described here there is only one potentially translatable RNA produced in addition to the RNA synthesised under the control of the T5 promoter: the bla mRNA.

The promoter of this transcriptional unit, P_bla, is, however, outcompeted by P_N25X/O by a factor of 25 (Deuschle and Bujard, in preparation). Thus, ~95% of the in vitro mRNA is P_N25X/O derived.

The plasmid family developed for this expression system has several advantages over commonly used expression vectors (Roberts et al., 1975; Paterson and Rosenberg, 1979; Melton et al., 1984; Krieg and Melton, 1984).

(i) The indicator genes encode proteins which are easily assayable and tolerated in large amounts by E. coli; they can therefore be brought under the control of highly efficient expression signals (Bujard et al., 1983).
(ii) The plasmids contain terminators of transcription in appropriate sites and can therefore be stably maintained upon integration of strong promoters (Gentz et al., 1981; Stueber and Bujard, 1982; Bujard et al., 1984).

(iii) In cloning experiments, bacterial cells harbouring the desired plasmids can be selected not only by monitoring ampicillin resistance, but the successful integration of a sequence between the promoter and the cat gene re-establishes chloramphenicol resistance in addition; thus the function of the transcriptional unit can be tested (prior phoshatase treatment of the cleaved cloning vehicle is of course required).

(iv) Sequences cloned in pDS5 will not be translated in E. coli since no ribosomal binding site is present. This permits the cloning of sequences coding for proteins poisonous to the E. coli cell. If fortuitous translation of such a sequence is suspected, transcription from P_N25X/O can be efficiently repressed by the lac repressor.

(v) There is no ATG between the transcription initiation site and the last integration site for foreign sequences; thus translation of the capped RNA will start at a site corresponding to the first ATG of the newly inserted DNA sequence and we have shown that the distance between the 5' end of the RNA and the first ATG can vary between 42 and 67 bp without affecting the efficiency of the system (Figure 2).

(vi) For translation in prokaryotic systems, foreign DNA sequences can be integrated into pDS5/3 derivatives which carry an efficient ribosomal binding site. Here the repressibility of transcription in vivo is useful since poisonous gene products can be kept at low enough levels to allow the cells to survive (Stueber et al., unpublished).

(vii) The DHFR protein, which can be produced in highly active form and yields of up to 50% of the total cellular protein (unpublished results) is a useful entity to construct fusion proteins and has the advantage of being considerably smaller than, e.g., β-galactosidase. As part of a fusion protein it can be translocated through membranes as demonstrated most elegantly in the following paper by Hurt et al. (1984).

A major advantage of the overall expression system reported here is the simplicity of the experimental procedure. The templates required can be isolated by the rapid 'mini prep' method according to Birnboim and Doly (1979). Aliquots of the transcription assay can be directly added to eukaryotic translation systems without any purification and, using [35S]methionine, labelled proteins can be synthesised with high specific activity. Aliquots of this assay can again directly be subjected to, e.g., SDS-PAGE. Moreover, due to the efficient labelling of the proteins autoradiograms can be obtained after <30 min of exposure. Thus, a complete experiment can be carried out in a day. A word of caution should be added here: preliminary data suggest that GC-rich sequences arising from GC-tailing can reduce the efficiency of translation in this expression system.

We have demonstrated the usefulness of the system with the coding sequences for three different proteins: dihydrofolate reductase, chloramphenicol acetyltransferase and lysozyme. Translation was specific and successful in the wheat germ as well as in the HeLa cell extract and the efficiency of translation of mRNA obtained by transcription-coupled capping in vitro was comparable with the translation of mRNA isolated from mammalian cells (MOPC 41). After the work reported here had been completed, another efficient but more tedious transcription system was reported (Melton et al., 1984; Krieg and Melton, 1984). Transcripts, obtained with SP6 RNA polymerase, are purified and capped by guanylyltransferase.

We believe that the method described here will prove to be useful for a variety of studies of protein structure and function. Using the lysozyme cDNA sequence we have shown that protein translation through membranes can be accomplished in the assay and further analysis of this process is being described in a forthcoming publication (Ibrahim et al., in preparation). Another interesting application of the system is demonstrated in the accompanying paper by Hurt et al. (1984). We are also confident that this expression system will be useful in the identification of cloned DNA sequences which code for proteins that are not tolerated in E. coli but against which antibodies are available.

Materials and methods

E. coli RNA polymerase, 7-methylguanosine 5'-monophosphate and 7mGpppA were from PL-Biochemicals, human placental RNase inhibitor was from BRL.

Construction of plasmids and preparation of the plasmid DNA

Construction of the various plasmids was performed by standard recombinant DNA methods and, with the exception of pDS5/4, will be described in detail elsewhere. pDS5/4 was obtained by inserting the HindIII fragment (Krieg et al., 1984) containing the entire coding region of lysozyme cDNA (Land et al., 1981) into the HindIII site of pDS5/1 (Figure 2).

Plasmid DNA was prepared either according to Birnboim and Doly (1979) or by the cleared lysate method (Clewel and Helinski, 1969) followed by CsCl/ethidium bromide equilibrium centrifugation (Radloff et al., 1967).

Both types of preparations were equally suitable for in vitro transcription and translation.

Coupled transcription-translation

The transcription-translation system was modified from Contreras et al. (1982), Roberts et al. (1975) and Müller et al. (1982). 2 μg of plasmid DNA were transcribed by 0.3 unit of E. coli RNA polymerase in a final volume of 10 μl containing 20 mM Hepes-KOH, pH 7.5, 10 mM Mg acetate, 200 mM K acetate, 0.2 mM spermidine, 5 mM diithiothreitol (DTT), 10 units of human placental RNase inhibitor (RNasin), 0.5 mM each of GTP, CTP, UTP, 5 μM ATP and 0.25 mM 7mGpppA. After incubation for 5 min at 37°C, 1 μl of 5 mM ATP was added and incubation continued for 15 min at 37°C. The mixture was put on ice and without further purification used in the translation assay. A typical 25 μl wheat germ translation assay contained 5 μl of the transcription mixture, 10 μl of wheat germ extract, and was adjusted to the following final concentrations: 20 mM Hepes-KOH pH 7.5, 110 mM K acetate, 2.8 mM Mg acetate, 1 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 80 μg/ml creatine phosphokinase, 5 μM S-adenosylmethionine, 20 μM of each of the 19 amino acids minus methionine, 10 μCi [35S]methionine. Where indicated, SRP (5 units per assay) and/or membranes from dog pancreas (0.05 AMU per assay) were included. The assays were incubated at 25°C for 60 min. Translation in the HeLa cell free system was done as described previously (Garoff et al., 1978).

Isolation of SRP (Walter and Blobel, 1983) and membranes from dog pancreas (Meyer et al., 1982), post-translational assays (Dobberstein and Blobel, 1977), SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Maizel, 1969) and fluorography (ENHANCE, NEN) were done as described.

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