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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 [^{35}S]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 $P_{N25X/O}$ and phage λ terminator t_0 (Figure 2). (ii) Downstream of t_0 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 β-lactamase gene (*bla*) are pBR322 derived. (iv) Using the proper *in vitro* transcription conditions, $P_{N25X/O}$ outcompetes the β-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 $P_{N25X/O}$ efficiently initiates transcripts with 7mGpppA resulting in capped mRNAs.

A foreign gene integrated downstream of the phage promoter can be expressed in *in vitro* systems with high efficiency using *Escherichia coli* RNA polymerase for transcription and purified pro- or eukaryotic cell lysates for translation. Supplying [^{35}S]methionine, the protein of interest can be obtained as labelled product of high specific activity, tested for different biological functions (e.g., translocation 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 t_0 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 pDS1 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

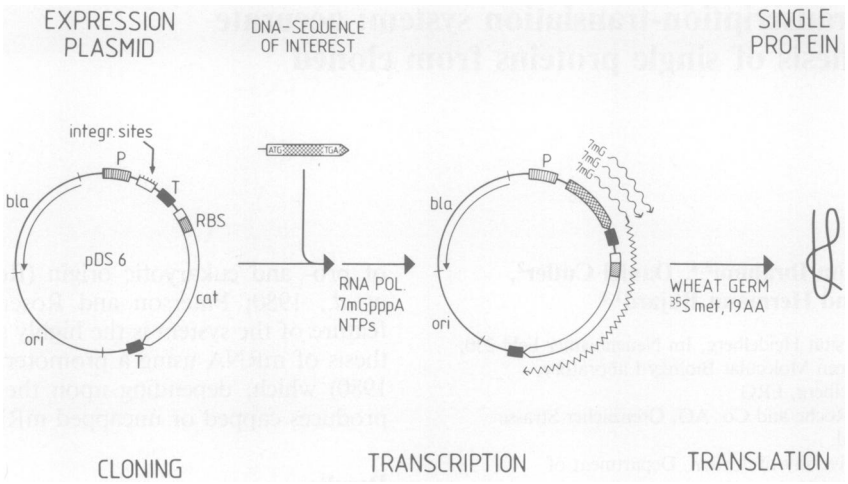

Fig. 1. Coupled transcription-translation system for production of a single protein species. DNA sequences of interest can be inserted at six unique restriction endonuclease cleavage sites of the expression plasmid pDS6 (Figure 2) between a strong coliphage T5-derived promoter (P) and an efficient transcriptional terminator (T). The integrity of the transcriptional unit constructed can be monitored by low level resistance to chloramphenicol due to the readthrough occurring at the terminator into the *cat* gene which lacks its promoter but still contains its ribosomal binding site (RBS). The readthrough transcripts are efficiently terminated at a second termination site (T) in front of the replication region (*ori*). Transformants can independently be selected for resistance to ampicillin conferred by the β-lactamase (*bla*) gene. Coupled transcription and translation: capped mRNA is produced by transcribing the plasmid with *E. coli* RNA polymerase (RNA POL) in the presence of 7mGpppA and the four ribonucleoside triphosphates (NTPs). The RNA is directly translated in a pro- or eukaryotic cell lysate (for example, the wheat germ system) in the presence of [^{35}S]methionine and the other 19 amino acids (AA) to virtually a single protein species.

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 *Xba*I to *Xho*I cleavage sites, which corresponds to the *Pvu*II/*Eco*RI 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_0 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 *rrnB* operon (Brosius *et al.*, 1981) downstream of the *cat* region. The only difference between the two plasmids is the presence of t_0 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

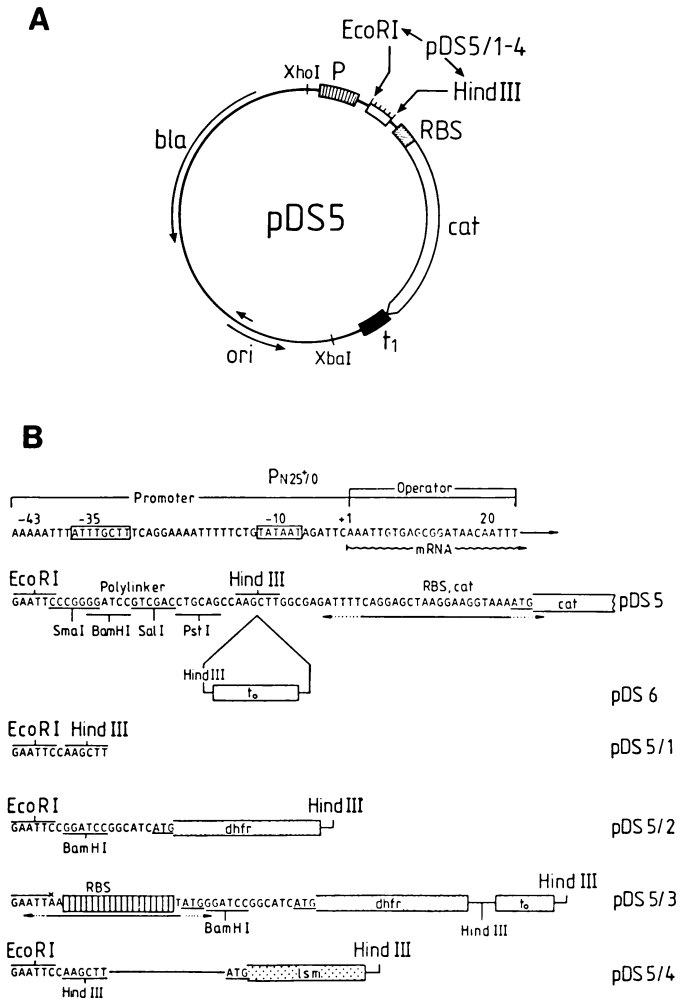

Fig. 2. Schematic description of plasmids. **(A)** Genetic and physical map of pDS5 and pDS6. Both plasmids contain the region between *Pvu*II (now *Xba*I) and *Eco*RI (now *Xho*I) of pBR322 carrying the replication region (*ori*) and the *bla* gene. The polylinker from pUC8 (Vieira and Messing, 1982) is placed between $P_{N25}x_{/O}$ (a fusion of coliphage T5 promoter P_{N25} 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 utilisation of the polylinker were removed from the plasmids. Transcripts from P are terminated *in vitro* and *in vivo* at terminator t_1 of the *rrnB* operon of *E. coli* and in addition also at terminator t_o of phage λ in the pDS6 system. The lower part of the figure shows the sequence between the −43 region of $P_{N25}x_{/O}$ of pDS5 and the start codon of the *cat* gene as well as the insertion site of the terminator t_o 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 *Hind*III site. pDS5/1: the 36-bp fragment of pDS5 is shortened to a 13-bp *Eco*RI-*Hind*III 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 t_o. pDS5/4: the cDNA for chicken lysozyme (lsm) has been inserted into the *Hind*III 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 *Pst*I and *Hind*II sites in the *bla* gene and the *Eco*RI site in the *cat* gene are still present. Furthermore, plasmids pDS5/1, 2 and 4 contain terminator t_o at the *Xba*I site (replacing t_1), whereas in pDS5/3 this termination signal is located between *dhfr* and *cat*.

achieved with an efficiency comparable with that obtained when an optimal concentration of mRNA from myeloma tumor MOPC 41 was translated under identical conditions.

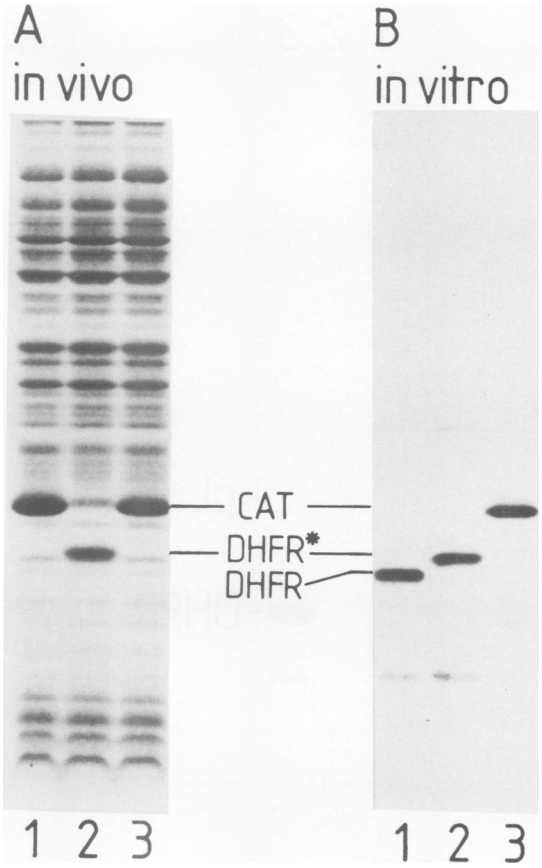

Fig. 3. Synthesis of DHFR and CAT encoded by plasmids pDS5/1−3. **(A)** *E. coli* cells (C600r⁻ △lacM15) harbouring the respective plasmids were grown overnight in Luria broth containing 100 μg of ampicillin/ml, harvested by centrifugation, solubilized in sample buffer and analysed by SDS-PAGE as described before (Stueber and Bujard, 1982). The Coomassie blue stained gels show the protein pattern of cells containing pDS5/2 **(lane 1)**, pDS5/3 **(lane 2)** and pDS5/1 **(lane 3)**. The positions of DHFR, its fusion derivative $DHFR^x$ (Figure 2) and of CAT are indicated. As can be seen, all these proteins are synthesised *in vivo* in high yields. **(B)** Plasmids pDS5/1−3 were transcribed with *E. coli* RNA polymerase in the presence of 7mGpppA. The resulting mRNAs were translated in a wheat germ cell-free system. The [^{35}S]methionine-labelled proteins were separated by SDS-PAGE and visualized by fluorography **(lane 1.** pDS5/2; **lane 2:** pDS5/3; **lane 3:** pDS5/1). About 10^5 c.p.m. were routinely obtained per 1 μl of translation mixture and 5 μl of the assay mixture were directly applied per slot of the gel. The dried gel was exposed to Kodak XAR5 film for 60 min.

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 *Hind*III site of pDS5/1. After transcription with *E. coli* RNA polymerase and translation 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 (RM_k) or of both SRP and RM_k.

The experiment depicted in Figure 5 shows that lysozyme is

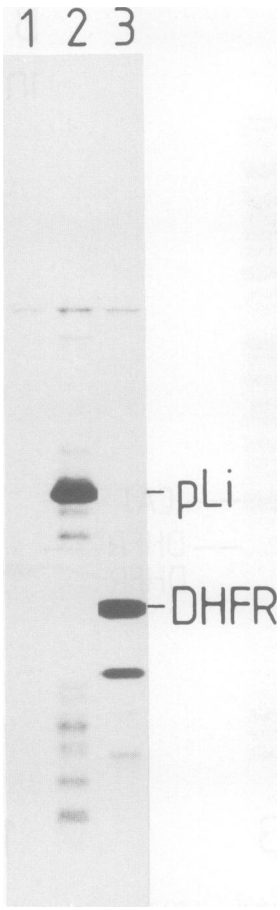

Fig. 4. Protein synthesis in the HeLa cell-free system. The translation system was supplemented with no RNA (**lane 1**), 10 μg/ml mRNA from MOPC 41 myeloma tumour (which produces mainly mRNA for the light chain of IgG, pLi) (**lane 2**) and an aliquot of an *in vitro* transcription assay with plasmid pDS5/2 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).

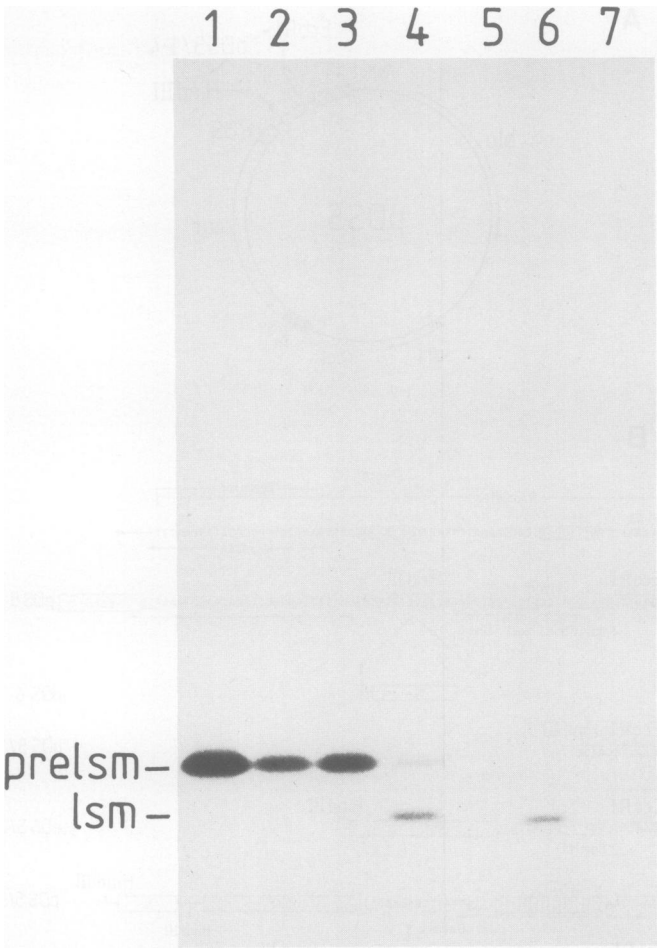

Fig. 5. *In vitro* synthesis and membrane translocation of lysozyme. Plasmid pSD5/4 containing the lysozyme cDNA sequence (Figure 2) was transcribed with *E. coli* RNA polymerase in the presence of 7mGpppA. The resulting RNA was translated in a wheat germ cell-free system and the labelled proteins were analysed by SDS-PAGE as described above. The different translation assays were supplemented for the translocation process as follows: **lane 1**, no addition; **lane 2**, +SRP; **lane 3**, +RM_k; **lane 4**, +SRP +RM_k. After translation in the presence of RM_k (**lane 5**) or SRP and RM_k (**lanes 6–7**), proteinase K (**lanes 5** and **6**) or proteinase K and Triton X-100 (**lane 7**) were added and the incubation was continued for 90 min on ice.

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* 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 phosphatase 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 [^{35}S]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 guanyltransferase.

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 translocation through membranes can be accomplished in the assay and further analysis of this process is being described in a forthcoming publication (Ibrahimi *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 *Hind*III fragment (Krieg *et al.*, 1984) containing the entire coding region of lysozyme cDNA (Land *et al.*, 1981) into the *Hind*III site of pDS5/1 (Figure 2).

Plasmid DNA was prepared either according to Birnboim and Doly (1979) or by the cleared lysate method (Clewell 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 dithiothreitol (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-adenosyl methionine, 20 μM of each of the 19 amino acids minus methionine, 10 μCi [^{35}S]methionine. Where indicated, SRP (5 units per assay) and/or membranes from dog pancreas (0.05 A_{280} 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.

Acknowledgements

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