[26] A T5 Promoter-Based Transcription–Translation System for the Analysis of Proteins *in Vitro* and *in Vivo*

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Expressing cloned genes in heterologous systems *in vitro* and *in vivo* has been instrumental in the identification and analysis of gene products and their derivatives.[1-4] Described here is a simple method that permits the expression of cloned sequences in *Escherichia coli* as well as in cell-free *in vitro* systems of eukaryotic (wheat germ, reticulocyte, HeLa cells) or prokaryotic (*E. coli*) origin using a single expression unit (Fig. 1). The essential element of this unit is a promoter derived from coliphage T5 that is utilized by *E. coli* RNA polymerase. It efficiently directs the synthesis of capped or uncapped mRNA *in vitro*.[5] Translation of such RNAs in the presence of [35S]methionine yields single proteins of such high specific activity and purity that they can be directly analyzed by gel electrophoresis without the necessity of prior immunoprecipitation.[5-9] Also described here is the application of this method to the study of structure/function relationships of proteins including (1) protein synthesis *in vivo* and *in vitro*, (2) the translocation of proteins into and through membranes, and (3) the interruption of translation at predetermined sites for the generation and characterization of truncated proteins.

[1] D. M. Coen, J. R. Bedbrook, L. Bogorad, and A. Rich, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5487 (1977).

[2] B. M. Paterson and M. Rosenberg, *Nature (London)* **279**, 692 (1979).

[3] M. Rosenberg, Y.-S. Ho, and A. Shatzman, this series, Vol. 101, p. 123.

[4] H.-L. Yang, L. Ivashkiv, H.-Z. Chen, G. Zubay, and M. Cashel, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7029 (1980).

[5] D. Stueber, I. Ibrahimi, D. Cutler, B. Dobberstein, and H. Bujard, *EMBO J.* **3**, 3143 (1984).

[6] E. C. Hurt, B. Pesold-Hurt, and G. Schatz, *EMBO J.* **3**, 3149 (1984).

[7] E. C. Hurt, B. Pesold-Hurt, K. Suda, W. Opplinger, and G. Schatz, *EMBO J.* **4**, 2061 (1985).

[8] A. Spena, E. Krause, and B. Dobberstein, *EMBO J.* **4**, 2153 (1985).

[9] J. A. Rothblatt and D. I. Meyer, *Cell* **44**, 619 (1986).
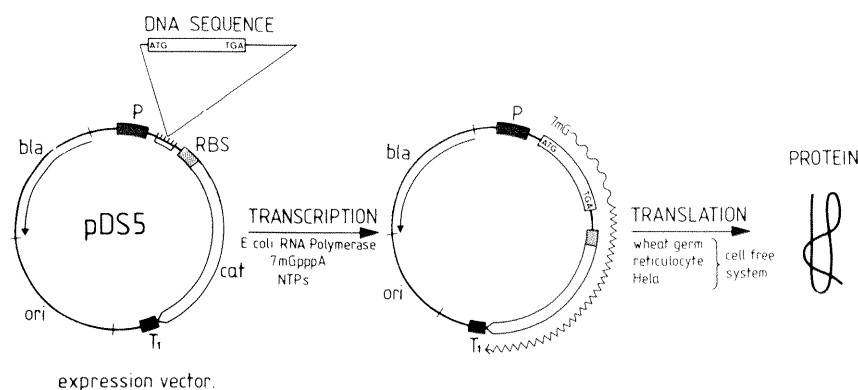
In vitro transcription - translation

FIG. 1. Coupled transcription–translation system for production of a single protein species. DNA sequences of interest can be inserted into the expression plasmid pDS5 at six unique restriction endonuclease cleavage sites (Fig. 2) located between a strong coliphage T5-derived promoter (P) and an efficient transcriptional terminator (T_1). The integrity of the transcriptional unit constructed can be monitored by resistance to chloramphenicol. This results from readthrough into the chloramphenicol acetyl transferase gene (*cat*) that lacks its promoter but still contains its ribosomal binding site (shaded boxes). The readthrough transcripts are efficiently terminated by T_1 in front of the replication region (*ori*). Transformants can be independently selected for resistance to ampicillin conferred by the β-lactamase gene (*bla*). Capped mRNA is produced by transcribing the plasmid with *E. coli* RNA polymerase in the presence of 7mGpppA and the four ribonucleoside triphosphates (NTPs). The RNA can be directly translated in the presence of [^{35}S]methionine in a prokaryotic or eukaryotic cell lysate.

Principles

A prerequisite for the translation of mRNA in eukaryotic systems is a 7-methylguanosine (7 mG) structure ("cap") at the 5' end.[10] For this reason, capping of the mRNA enhances translation several fold in all eukaryotic cell-free systems. The low level of translation observed with uncapped mRNA is most likely due to a weak intrinsic capping activity of the lysates.[1] Capped or uncapped mRNA can be obtained readily *in vitro* using *E. coli* RNA polymerase and some promoters of coliphage T5 as a transcription system. These promoters belong to the strongest transcription initiation signals of the *E. coli* system *in vitro* and *in vivo*.[11] When cloned into a plasmid vector, they outcompete any common plasmid-

[10] A. J. Shatkin, *Cell* **40**, 223 (1985).
[11] R. Gentz and H. Bujard, *J. Bacteriol.* **164**, 70 (1985).

encoded promoter. Competition between the T5 promoter P_{N25} and P_{bla}, the promoter of the β-lactamase (bla) gene, results in more than 95% of the RNA chains being initiated by P_{N25} in vivo and in vitro. As has been shown previously for the promoter P_L from bacteriophage λ,[12] several of the strong T5 promoters, such as P_{N25}, accept the capping dinucleotide 7mGpppA as starting nucleotide for RNA synthesis even in the presence of high concentrations of ribonucleoside triphosphates.[5] Thus, specific mRNAs in capped or uncapped form are obtained in high yields. These RNAs can be translated directly in a cell-free system. Another widely used transcription system is based on the selectivity of the phage SP6-specified RNA polymerase that exclusively utilizes phage SP6 promoters.[13] The promoter/RNA polymerase combination described here has, however, a significant advantage. The identical expression unit can be used in vitro and in vivo since the same enzyme is acting in both environments. Furthermore, it appears that E. coli RNA polymerase reads through sequences which may terminate transcription elongation complexes of other enzymes.[14]

The Vector System

Plasmid pDS5 and its derivatives[5] are members of a plasmid family developed for the study of transcriptional signals.[15] The essential properties of the pDS5 system can be summarized as follows (Fig. 2 and Table I):

It contains a fusion between the strong coliphage T5 promoter P_{N25} and the lac operator (termed P_{N25}/O). Transcription from this promoter can therefore be controlled by the lac repressor (lac i product).

A polylinker adjacent to P_{N25}/O permits the insertion of DNA sequences at various sites.

Transcripts from P_{N25}/O up to the end of the polylinker do not contain an ATG codon; thus, capped RNAs will start translation from the first AUG of the insert.

The polylinker is followed by an indicator gene, the sequence for chloramphenicol acetyltransferase (cat). This gene is promoter free but carries its own ribosomal binding site.

[12] R. Contreras, H. Cheroutre, W. Degrave, and W. Fiers, Nucleic Acids Res. **10,** 6353 (1982).
[13] P. A. Krieg and D. A. Melton, Nucleic Acids Res. **12,** 7057 (1984).
[14] M. Chamberlin, K. Kingston, M. Gilman, J. Wiggs, and A. DeVera, this series, Vol. 101, p. 540.
[15] D. Stueber and H. Bujard, EMBO J. **1,** 1399 (1982).
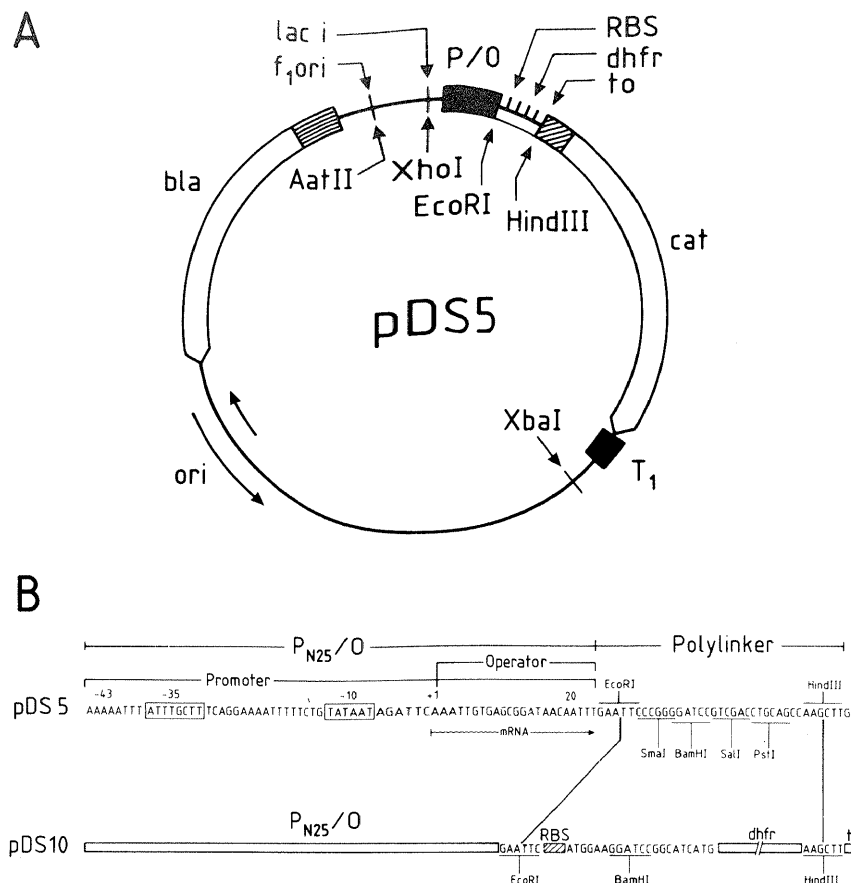
FIG. 2. Schematic description of the plasmid system. (A) Outline of plasmid pDS5, which contains the following essential elements[5]: P/O, a fusion between coliphage T5 promoter P_{N25} and the *E. coli lac* operator; a polylinker from pUC8 [J. Vieira and J. Messing, *Gene* **19**, 259 (1982)]; the *cat* gene with its ribosomal binding site (diagonally shaded box), and terminator T_1. The region between the *Xba*I and *Xho*I site corresponds largely to the sequences of pBR322 between the *Pvu*II and *Eco*RI site containing the *colE1* origin of replication and the *bla* gene with its promoter and its ribosomal binding site (horizontally shaded box). The *Hinc*II and the *Pst*I site in the *bla* as well as the *Eco*RI site in the *cat* gene were removed. Several unique cleavage sites were used to integrate ribosomal binding sites (RBS), sequences of interest such as mouse dihydrofolate reductase (*dhfr*), or terminators such as t_0 from coliphage λ. For suppression of the promoter, *lac* repressor can be provided by the *lac i* gene inserted at the *Xho*I site. Finally, to facilitate sequencing or site-directed mutagenesis, the constructs can be converted into their single-stranded form utilizing the replication origin of the single-stranded DNA phage f1 integrated at the *Aat*II site.[16] Some of the key constructs of this system are summarized in the Table I. (B) The nucleotide sequence of the promoter/operator/polylinker region of pDS5. The essential features of this sequence are shown. Conversion of pDS5 to pDS10 by the integration of the ribosomal binding site, the *dhfr* coding sequence, and terminator (t_0) into the polylinker is shown. The *Hind*III site between the t_0 and *cat* has been removed in pDS10.

TABLE I

NOMENCLATURE OF SOME OF THE AVAILABLE
PLASMID CONSTRUCTS[a]

	t_0	dhfr	RBS	lac i	f1 ori
pDS5	−	−	−	−	−
pDS6	+	−	−	−	−
pDS7	−	+	−	−	−
pDS8	+	+	−	−	−
pDS9	−	+	+	−	−
pDS10	+	+	+	−	−
pDS20	+	−	−	+	−
pDS21	−	+	+	+	−
pDS30	−	−	−	−	+
pDS31	+	−	−	−	+
pDS32	+	−	−	+	+

[a] Some members of the pDS plasmid family
used in our studies can be characterized by
combining pDS5 (Fig. 2) with the elements de-
picted at the top of the table. All of the combi-
nations indicated with + have been con-
structed, and were designated as shown in the
left-most column. The pDS20 series contains
the lac i gene of E. coli on a 1200-bp fragment
and the pDS30 series carries the replication
origin of coliphage f1 in a 570-bp fragment.
None of these elements contains any of the
unique cleavage sites of pDS5. Some of these
plasmids replace earlier constructs. Thus,
pDS7 and pDS10 correspond to pDS5/2 and
pDS5/3 published previously.[5] In the new
constructs the HincII, PstI, and EcoRI cleav-
age sites were removed from the bla and cat
gene, respectively. Depending on the ribo-
some binding site (RBS) used, protein yields
can vary up to 20-fold.

Between the cat gene and the origin of replication, the terminator T_1 of
the rrnb operon has been inserted to prevent deleterious transcriptional
readthrough into the replication region.[15]

Integration of the terminator t_0 from phage λ in front of the cat gene
yields pDS6. This terminator reduces transcription into cat 10-fold,
thereby preventing growth, inhibiting overproduction of CAT protein, but
allowing sufficient synthesis of this protein to select for intermediate lev-
els of chloramphenicol resistance.

Insertion of the coding sequence for dihydrofolate reductase of the mouse (pDS7–pDS10; Table I) provides a useful indicator product, which has been utilized in various studies, including the translocation of protein across membranes.[5-7]

For expression in prokaryotic systems, ribosomal binding sites (RBS) may be inserted in front of the cDNA sequence of interest, and protein synthesis mediated by such signals can be studied *in vivo* and *in vitro*.

To control P_{N25}/O, *in vivo*, the *lac i* gene coding for repressor protein can be inserted into the unique *XhoI* site, yielding plasmids of the pDS20 series.

Similarly, insertion of the replication origin of coliphage f1 into the *AatII* site results in vectors of the pDS30 series. Superinfection of cells harboring these plasmids with phage f1 converts the plasmid into its single-stranded form, which facilitates sequence analysis and site-directed mutagenesis of the constructs.[16]

Some of the constructs prepared in our laboratory are summarized in Table I and the essential nucleotide sequences of pDS5 and pDS8 are depicted in Fig. 2B. All these plasmids are stable, have normal intracellular copy numbers,[15] and can be isolated by standard methods.

Transcription and Transcription-Coupled Capping *in Vitro*

For achieving selective transcription, it is necessary to avoid a large excess of RNA polymerase and to have a rather high ionic strength in the transcription assay. With promoters like P_{N25} a ratio of RNA polymerase to promoter of 5 : 1 and a salt concentration equivalent to 0.2 M potassium acetate is optimal. The specificity and efficiency of transcription can be examined by electrophoretic analysis of the RNA (Fig. 3A). Successful capping can be similarly tested since incorporation of 7mGpppA alters the electrophoretic mobility of short transcripts sufficiently to be detected (Fig. 3B).[11]

Procedure

Transcription in Vitro. Standard *in vitro* transcription assays are carried out in a volume of 100 μl containing 20 mM HEPES–KOH (pH 7.9), 200 mM kalium acetate (KOAc), 10 mM magnesium acetate [Mg(OAc)$_2$], 5 mM DTT, 0.2 mM spermidine, 0.1 mM EDTA, 2 U of human placental RNase inhibitor, 0.5 mM each of ATP, GTP, and CTP, 100 μM UTP, 0.5 μM [α-^{32}P]UTP (3000 Ci/mmol), and 1 μg of plasmid DNA. The reactions

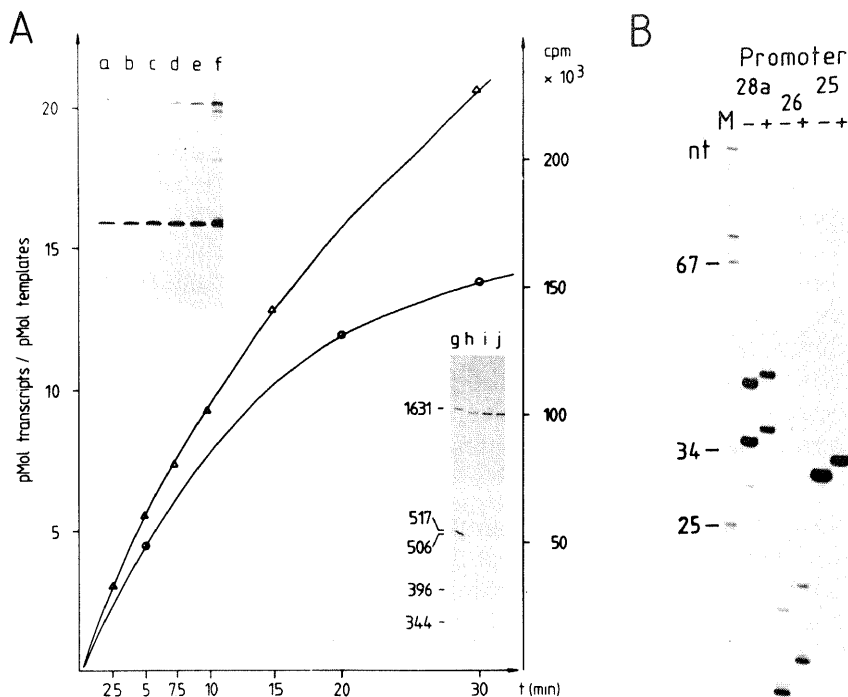[16] G. P. Dotto, V. Enea, and N. D. Zinder, *Virology* 114, 463 (1981).

FIG. 3. Transcription and transcription-coupled capping *in vitro*. (A) Time course of *in vitro* RNA synthesis directed by coliphage T5 promoters P_{N25} and P_{N26}.[11] RNA synthesis was allowed to proceed in 150 mM KCl in the presence of [α-^{32}P]UTP. The templates were pDS1 derivatives linearized to produce runoff transcripts of 192 ($\triangle$ and upper left insert) or 1600 nucleotides (nt) ($\bigcirc$, lower right insert) in length, respectively. Samples of the reaction mixture were withdrawn at the times indicated and divided into two fractions, of which one was used to monitor the acid-precipitable counts ($\bigcirc$, $\triangle$) and the other for analysis by PAGE (inserts). In the upper left insert, lanes a–f show the electrophoretic analysis of samples taken at 2.5 through 30 min; lanes h–j of the lower right insert depict the corresponding analysis of samples taken at 5, 20, and 30 min, respectively. Lane g denotes a size marker. (B) Assay for transcription-coupled capping *in vitro*. Plasmid pDS1 derivatives containing coliphage T5 promoters P_{K28a}, P_{N26}, and P_{N25} were transcribed with (+) and without (−) 7mGpppA in the presence of methyl-CTP instead of CTP. The labeled transcripts were separated by 7 M urea PAGE and visualized by autoradiography. For P_{K28a} and P_{N26} the first and second C in the coding sequence determines the length of the two transcripts. For P_{N25} there is only one transcript. The retardation of the capped RNAs is clearly visible.

are initiated by the addition of 0.2 U of *E. coli* RNA polymerase and stopped after incubation for 30 min at 37° by the addition of EDTA to 40 mM. The sample is extracted twice with phenol and the RNA is precipitated with ethanol. The RNA pellet is redissolved in TE buffer [10 mM

Tris · HCl (pH 7.9), 1 mM EDTA], and after the addition of three volumes of 90% formamide, heated to 90° for 30 sec and analyzed by PAGE in 7 M urea.[17]

Transcription-Coupled Capping in Vitro. Several T5 phage promoters are capable of initiating transcription *in vitro* with 7mGpppA.[11] Successful incorporation of this dinucleotide can be monitored indirectly by translating the resulting RNA in a eukaryotic translation system. Direct proof of capping is obtained, however, by generating short, capped transcripts that exhibit a different electrophoretic mobility when compared to their uncapped form. Short transcripts are produced by replacing CTP with methyl-CTP (200 μM final concentration) and highly efficient capping occurs when the concentration of ATP is lowered to 50 μM and 7mGpppA is included (final concentration, 250 μM) in a standard transcription assay. After 5 min at 37° the ATP concentration is adjusted to 500 μM and incubation is continued for 30 min. Methyl-CTP stops RNA synthesis at the first or second C residue, resulting in one or two RNA species (Fig. 3B). For analysis by PAGE (7 M urea, 20% acrylamide, 50 V/cm, 3 hr), transcripts are purified as described above.

Transcription–Translation in Vitro. Transcripts produced as described above can either be purified prior to translation or can be directly transferred from the transcription into the translation assay. In the latter case it is important to perform the transcription in a small volume, since only one-fifth of the volume of the translation assay is available for the addition of transcripts. This ensures that an optimal amount of transcript will be present during translation, while the concentration of unincorporated 7mGpppA is low enough to avoid competition with translation initiation.

Reagents and Solutions

Transcription premix (for 10 transcription assays, each in a final volume of 10 μl of mix): 2 μl of 1 M HEPES–KOH (pH 7.9); 5 μl of 4 M KOAC; 1 μl of 1 M Mg(OAc)$_2$; 1 μl of 20 mM spermidine; 1 μl of 0.5 M DTT; 1 μl each of 50 mM of GTP, UTP, and CTP; 1 μl of 5 mM ATP; 1 μl of 0.5 mM S-adenosylmethionine; 5 μl of 5 mM 7mGpppA; 1 μl of 20 U/μl human placental ribonuclease inhibitor; 2 μl of 2 U/μl *E. coli* RNA polymerase (Pharmacia-PL, Uppsala); and H$_2$O to a final volume of 40 μl

Template to be transcribed: plasmid DNA (0.2–2 μg/μl) in TE buffer

[17] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1982.

ATP (5 mM) for the chase reaction

Translation premix (for 10 assays, each in a final volume of 25 μl):
mix 7.5 μl of 1 M HEPES–KOH (pH 7.6); 5 μl of 50 mM ATP; 5 μl
of 500 mM creatine phosphate; 0.5 μl 4 mg/ml creatine phospho-
kinase; 5 μl of a mixture of 19 amino acids minus methionine (1
mM each); 25 μl of [^{35}S]methionine (800 Ci/mmol, 10 mCi/ml); 100
μl of wheat germ extract (in 10 mM Tris–OAc (pH 7.6); 50 mM
KCl; 1 mM Mg(OAc)$_2$; 6.25 μl of 2 M KOAc; add H$_2$O to 160 μl.

Procedures

Transcription. For a transcription assay in a final volume of 10 μl, mix
4 μl of the transcription premix with 1–3 μg of plasmid DNA and adjust
the volume to 9 μl with H$_2$O. Incubate at 37° for 5 min. Then add 1 μl of 5
mM ATP and continue the incubation for a total of 30 min. Put the sample
on ice if directly used for translation or store for later use at −20°. The
final concentrations of the components are 20 mM HEPES–KOH (pH
7.9); 200 mM KOAc; 10 mM Mg(OAc)$_2$; 0.2 mM spermidine; 5 mM DTT;
0.5 mM each of GTP, UTP, and CTP; 0.05 mM ATP; 5 μM S-adeno-
sylmethionine; 0.25 mM 7mGpppA; 0.2 U/10 μl human RNase inhibitor;
0.3–0.5 U of *E. coli* RNA polymerase.

Translation. For a translation assay in a final volume of 25 μl, combine
16 μl of the translation premix with 5 μl of the transcription assay and add
H$_2$O to a final volume of 25 μl. Incubate at 25° for 60 min, then store the
samples on ice and process for SDS–PAGE and fluorography.

Applications

Coupled *in vitro* transcription–translation has successfully been ap-
plied in the identification and characterization of proteins encoded in
cloned sequences.[1,4,12] In the following discussion we describe three ex-
amples which show the potential of this system.

Differential Expression of DNA Sequences *in Vitro* and *in Vivo* (*E. coli*)

It cannot be predicted with certainty whether a particular DNA se-
quence cloned into a bacterial expression unit will be expressed. There
are a number of reasons why such an expected gene product might not be
identifiable, including higher order structures of the mRNA, which are not
compatible with the bacterial expression machinery; instability of the
mRNA or the protein; and toxicity of the gene product frequently ob-
served with membrane proteins. Using an *in vitro* expression system as
described above, the identical expression unit as used in *E. coli* can be

tested *in vitro* using different translational systems. This permits to determine where the *in vivo* expression might be limited, since RNA half-life and protein degradation can be controlled *in vitro*.

A comparison of the expression of *dhfr* and *cat* in *E. coli* and in a eukaryotic cell-free system is shown in Fig. 4. While translation in *E. coli*
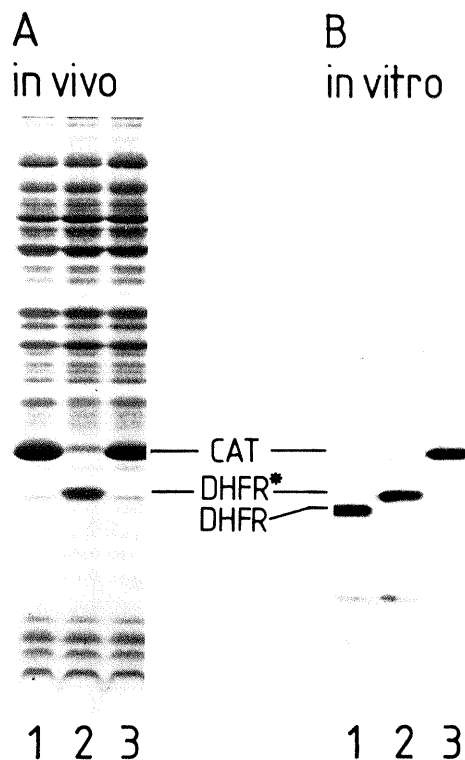

FIG. 4. Synthesis of DHFR and CAT *in vivo* and *in vitro*.[5] (A) *Escherichia coli* cells (C600r⁻, *lac*M15 deletion strain DZ 291) harboring the plasmid pDS5, pDS7, or pDS10, respectively, were grown overnight in Luria broth containing 100 μg/ml of ampicillin, harvested by centrifugation, solubilized in sample buffer, and analyzed by SDS–PAGE. The Coomassie blue-stained gels show the protein pattern of cells containing pDS7 (lane 1), pDS10 (lane 2), and pDS5 (lane 3). The positions of DHFR, of its fusion derivative DHFR* (Fig. 2), and of CAT are indicated. All these proteins are synthesized *in vivo* in high yields. (B) Plasmids pDS5, pDS7, and pDS10 were transcribed *in vitro* with *E. coli* RNA polymerase in the presence of 7mGpppA. The resulting mRNAs were translated in a wheat germ cell-free system. The [³⁵S]methionine-labeled proteins were separated by SDS–PAGE and visualized by fluorography. About 1×10^5 cpm were routinely obtained per 1 μl of translation mixture, and 5 μl of the assay was applied per slot of the gel. The dried gel was exposed to Kodak XAR-5 film for 60 min.

depends on the presence of a ribosomal binding site within a polycistronic mRNA, translation of capped mRNA starts in an eukaryotic cell-free system at the first AUG.[5] Expression *in vitro* of various DNA sequences demonstrates (Fig. 5) how the pDS5 system can be utilized for a rapid examination of the coding capacity and the integrity of new constructs.

A typical example for differences of the *in vivo* (*E. coli*) and *in vitro* expression are the two related DNA sequences coding for IFN-γ and a fusion between a signal peptide and IFN-γ, termed S-IFN-γ. Plasmid-encoded IFN-γ is expressed in bacteria and also in eukaryotic cell-free system. By contrast, S-IFN-γ is not expressed in bacteria but it is synthesized in the eukaryotic and the prokaryotic cell-free system (Fig. 6). We therefore conclude that all expression signals in the S-IFN-γ construct are functional, and that S-IFN-γ or its mRNA may be rapidly degraded in
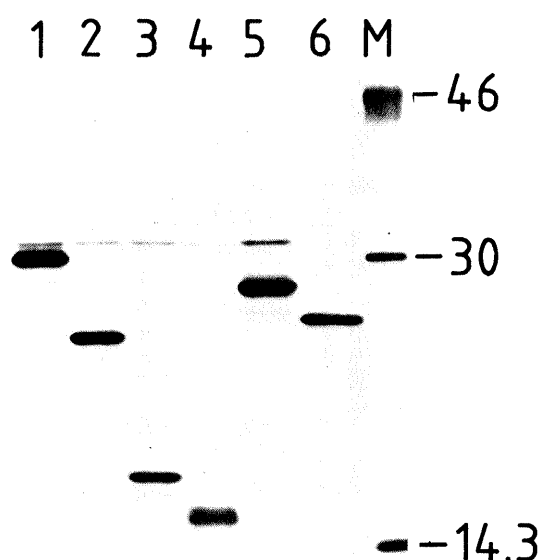

Fig. 5. General applicability of the pDS5 system. The pDS5-derived vectors containing various cDNA inserts in the polylinker region were tested for the expression *in vitro* of the respective proteins using a wheat germ translation assay. The cDNAs code for the following proteins: lane 1, ENV(80)DHFR, a fusion protein between DHFR and an 80-amino acid sequence of p41 of LAV/HTLV-III; lane 2, DHFR of the mouse; lane 3, γ-interferon (IFN-γ); lane 4, platelet-derived growth factor (PDGF); lane 5, a signal peptide/CAT fusion (S-CAT); lane 6, a signal peptide/DHFR fusion (S-DHFR). The faint band with a molecular weight of 31,000, present in all lanes, is β-lactamase. The lane M contains molecular-weight markers.
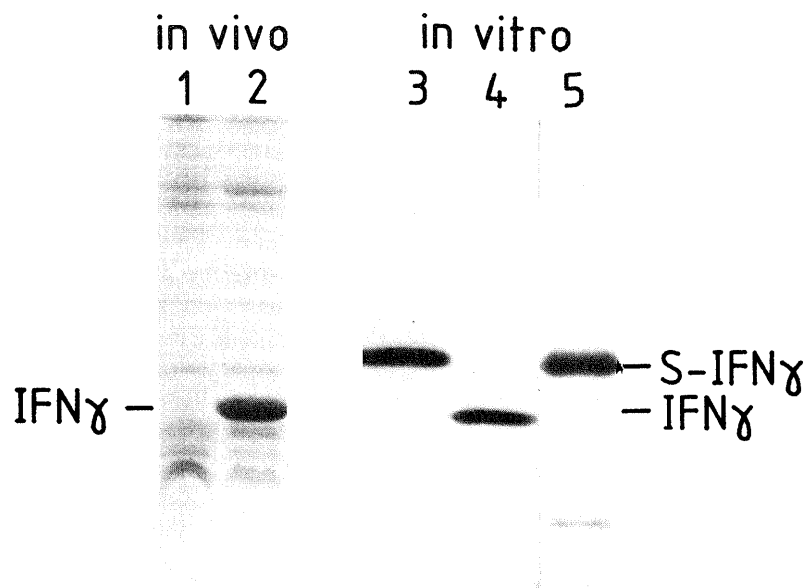
FIG. 6. Differences between *in vivo* and *in vitro* protein synthesis. Derivatives of pDS6 containing either the coding sequence of IFN-γ or of a signal peptide/IFN-γ fusion (S-IFN-γ) were expressed in *E. coli* (lanes 1 and 2) or *in vitro* in a wheat germ (lanes 3 and 4) as well as in an *E. coli* cell-free extract (lane 5). The identical RBS precedes the respective coding sequences in both plasmid constructs.

bacteria. It was actually confirmed that S-IFN-γ, in contrast to IFN-γ, is rapidly degraded in *E. coli.* by endogenous proteases (unpublished observation).

Translocation of Proteins across Membranes *in Vitro*

Several properties of signal sequences that determine translocation of proteins across or insertion into membranes have successfully been analyzed in cell-free systems.[18] In these systems, proteins are translated from mRNAs encoding a protein destined for a cellular organelle such as the rough endoplasmic reticulum (ER), mitochondria, chloroplasts, or peroxisomes. When organelles are present during translation or in some cases even after translation, uptake of the newly synthesized protein into the organelle occurs, provided that it contains a functional, organelle-specific signal sequence.[18,19]

[18] T. A. Rapoport and M. Wiedmann, *Curr. Top. Membr. Transp.* **24**, 1 (1985).
[19] G. Blobel and B. Dobberstein, *J. Cell Biol.* **67**, 835 (1975).

428   MISCELLANEOUS METHODS   [26]

A test system for the translocation of a secretory protein into microsomal vesicles derived from ER membranes is described here. It involves (1) translocation arrest of a secretory protein by a signal recognition particle (SRP),[20] (2) release of this arrest by rough microsomal (RM) membranes from dog pancreas, and (3) proteolysis of RM membranes in order to demonstrate the location of the newly synthesized polypeptide chain. Nascent secretory proteins are translocated across the ER membrane by a receptor-mediated process involving SRP[20] and docking protein (DP).[21] SRP interacts with the nascent protein chain, thereby causing arrest of chain elongation in a wheat germ cell-free translation system which is released by DP, a protein associated with the rough ER.[20,21]

In Fig. 7 we show, as an example, the translocation of the hybrid secretory protein S-DHFR across ER membranes. Plasmid pDS-*s-dhfr* encodes a protein composed of the signal peptide of the enterotoxin B and the cytoplasmic protein DHFR (S-DHFR). After transcription of pDS-*s-dhfr* with *E. coli* RNA polymerase, the resulting mRNA was translated in the wheat germ cell-free system in the absence or presence of SRP and RM (Fig. 7). The ability to obtain a single mRNA species is of particular importance in the analysis of SRP-mediated arrest of nascent chain elongation, as otherwise the arrested polypeptide cannot be demonstrated (see Fig. 7, lanes 1 and 2).

Reagents and Solutions

Translation premix: as described above, but KOAc is replaced by H_2O. KOAc will be added with the SRP or the SRP buffer.

Signal recognition particle: 0.5–1 A_{280} U/ml, sucrose gradient purified, in SRP buffer.[20]

SRP buffer: 50 mM triethanolamine–OAc (TEA–OAc) (pH 7.5); 650 mM KOAc; 5 mM Mg(OAc)$_2$; 5 mM DTT.

RM from dog pancreas (40 A_{280} U/ml) washed with 0.5 M KCl and treated with micrococcal nuclease in RM buffer (RM$_{KN}$).[22]

RM buffer: 50 mM TEA–OAc (pH 7.5); 50 mM KOAc; 5 mM Mg(OAc)$_2$; 5 mM DTT; 2 mM EGTA; 0.25 M sucrose.

50 mM 7-methylguanosine (7 mG) in 20 mM HEPES-KOH (pH 7.9).

5 mg/ml proteinase K.

5% Nonidet P-40.

Procedure

For a translation assay in a final volume of 25 μl prepare the translation premix devoid of KOAc. Combine 16 μl of this mixture with 5 μl of

[20] P. Walter and G. Blobel, this series, Vol. 96, p. 682.
[21] D. I. Meyer, E. Krause, and B. Dobberstein, *Nature* (*London*) **297**, 647 (1982).
[22] H. Garoff, K. Simons, and B. Dobberstein, *J. Mol. Biol.* **124**, 587 (1978).

A

pDS-s-dhfr

P RBS s   dhfr   pDS
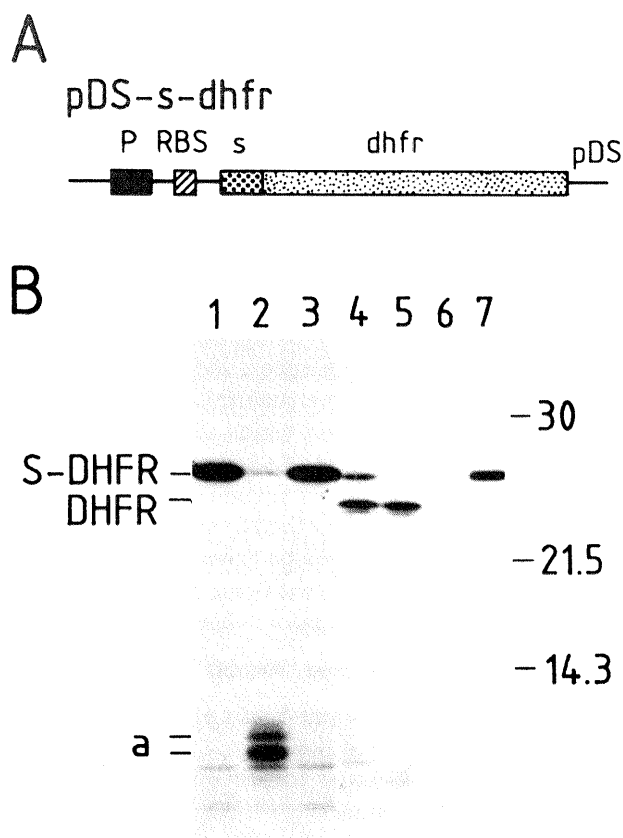
B

1 2 3 4 5 6 7

-30

S-DHFR —
DHFR —

-21.5

-14.3

a —

FIG. 7. Test for the function of a signal sequence. Plasmid pDS-s-dhfr contains the signal sequence of the enterotoxin B fused to the sequence coding for DHFR. The ability of the signal sequence to translocate DHFR across ER membranes was tested in a cell-free membrane translocation system. pDS-s-dhfr was transcribed and the resulting mRNA translated in a wheat germ cell-free system with the following additions: lane 1, none; lane 2, SRP; lane 3, RM_{KN}; lane 4, SRP and RM_{KN}; in lanes 5 and 6 proteinase K or proteinase K and Nonidet P-40 were added and incubation was continued for 10 min at 25°. Lane 7 shows S-DHFR immunoprecipitated with an anti-DHFR antibody; a, arrested peptide.

the transcription assay, 2 μl of SRP or SRP buffer, and 1–2 μl of RM_{KN} or RM buffer and adjust the volume to 25 μl with H_2O. Incubate for 60 min at 25°. To test the SRP dependence of membrane translocation, translation has to be synchronized.[20] This is achieved by adding 2 μl of 50 mM 7-methylguanosine (7mG) to a 25-μl translation assay 5 min after the start of translation. The 7mG interferes with polypeptide chain initiation. The SRP-mediated block of elongation is relieved by adding RM_{KN} after 30 min of SRP-arrested translation. After completion of translation, mem-

brane insertion or translocation of nascent proteins is tested as follows: RM-containing samples are treated with proteinase K (0.5 mg/ml) or proteinase K (0.5 mg/ml)/Nonidet P-40 (0.5%) for 10 min at 25°. Proteins translocated into RM vesicles are protected against protease attack. When, however, the membrane barrier is destroyed by detergent, all proteins should be digested.

Synthesis of Truncated Proteins in Vitro

The synthesis of defined fragments of polypeptides is of interest for various structural and functional investigations, such as assignment of active sites to specific protein sequences, identification and mapping of epitopes, and of topogenic peptide structures.

Two methods are described here which yield C terminally truncated polypeptide chains. In the first, the DNA template is shortened by restriction endonucleases,[23] whereas in the second, oligodeoxynucleotides are used to arrest translation.[24] Both methods yield polypeptides, the majority of which are released from the translational complex as peptidyl-tRNA. Free polypeptides are obtained upon prolonged incubation.[24]

Synthesis of Protein Fragments from "Runoff" Transcripts

In this procedure the cDNA cloned into pDS-type vectors is cut with restriction enzymes that cleave at the desired positions within the coding sequence. Sticky ends are blunted to prevent readthrough at annealed sites.[17] Runoff transcripts obtained with E. coli RNA polymerase are translated in a cell-free system. An example of this approach is shortened prelysozyme molecules, which were used to determine the minimal length of a protein that can still be translocated across ER membranes (Fig. 8).[23]

Reagents and Solutions. Relevant restriction enzymes, T4 DNA polymerase, or Klenow fragment of E. coli polymerase are used.

Procedure

Digest plasmid DNA with the relevant restriction endonuclease (make sure that the enzyme does not cleave between the promoter and the 5' untranslated region).

Test for completion of digestion by agarose gel electrophoresis; if necessary, continue digestion.

[23] I. Ibrahimi, D. Cutler, D. Stueber, and H. Bujard, *Eur. J. Biochem.,* **155,** 571–576 (1986).
[24] M.-T. Haeuptle, R. Frank, and B. Dobberstein, *Nucleic Acids Res.* **14,** 1427 (1986).
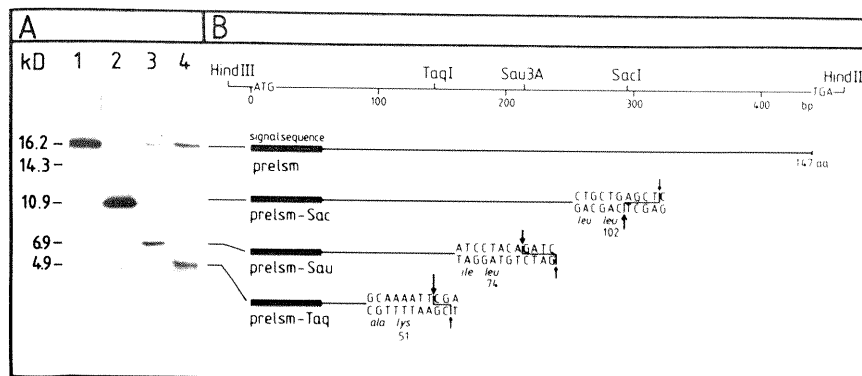
FIG. 8. Synthesis of truncated proteins from runoff transcripts of various lengths.[23] (A) A pDS5 derivative containing the complete coding region of prelysozyme was cleaved with the restriction enzymes SacI, Sau3A, and TaqI [see (B)]. The plasmid and the fragments were then used in the transcription–translation assay. The total ^{35}S-labeled translation products were analyzed by SDS–PAGE. The autoradiogram shows the following proteins: lane 1, prelysozyme (prelsm); lane 2, prelsm/Sac; lane 3, prelsm/Sau; lane 4, prelsm/Taq. (B) Schematic representation of prelsm and of the restriction enzyme cleavage sites used for the generation of the different runoff transcripts. Protruding ends of the transcripts were blunted before transcription. The sizes of prelsm and of the various truncated proteins are indicated.

If sticky ends are generated as a result of endonuclease digestion, then blunt these with T4 DNA polymerase or with a Klenow fragment of *E. coli* DNA polymerase.[17]

Extract with phenol : chloroform.

Precipitate the DNA with 2 volumes of ethanol; redissolve pelleted DNA in TE buffer.

Transcribe and translate.

Oligodeoxynucleotide-Mediated Arrest of Translation

Polypeptides truncated at any given site can be obtained if oligodeoxynucleotides complementary to the corresponding mRNA sequence are included in the translation mixture.[24] An endogenous ribonuclease H-like activity cleaves the mRNA at the site of the DNA/RNA hybrid, yielding transcripts of the expected size (see Fig. 9). Wheat germ lysates usually contain sufficient endonucleolytic activity to efficiently cleave such mRNAs. By contrast, reticulocyte lysates have to be complemented with exogenous ribonuclease H (approximately 0.2–1 U/25 μl translation assay).
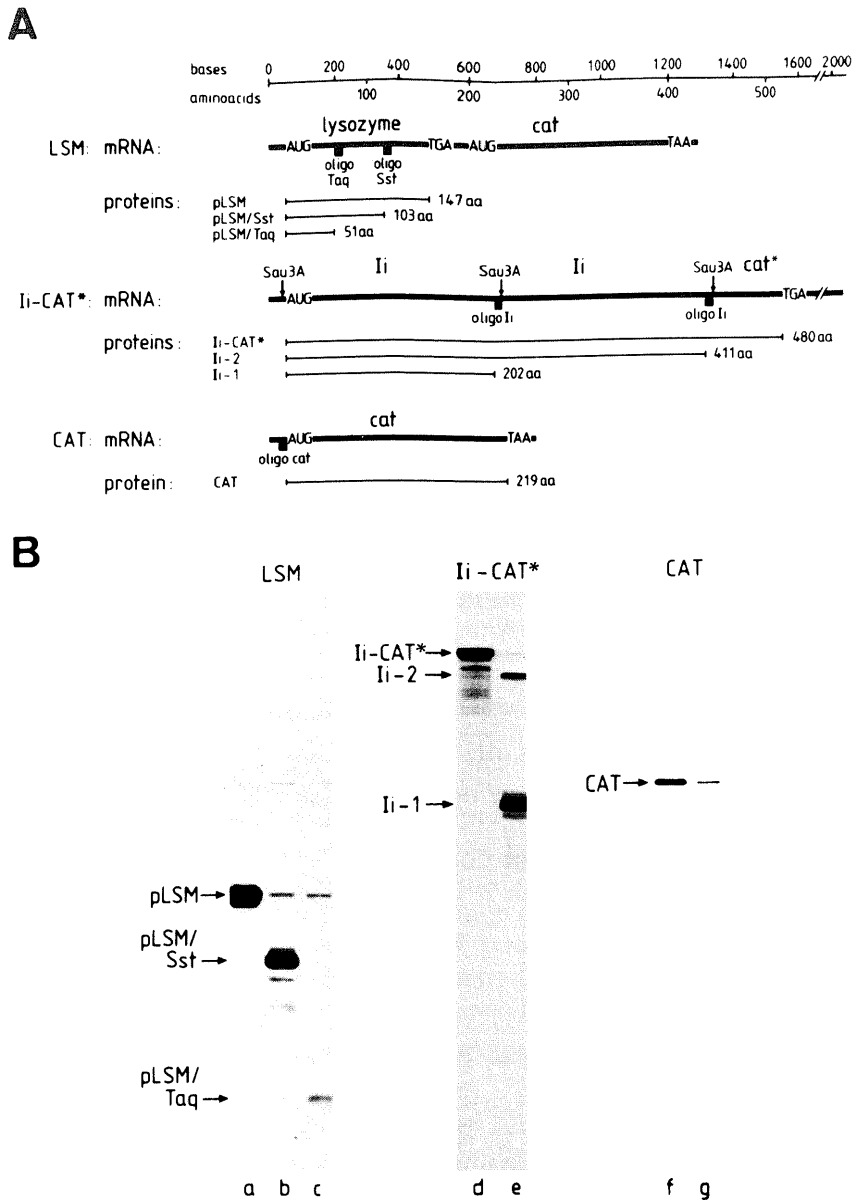
FIG. 9. Synthesis of truncated proteins by oligodeoxynucleotide-mediated translation arrest.[24] (A) Schematic outline of three mRNAs, positions of the complementary oligodeoxynucleotides (■) and expected translation products. All RNAs were transcribed from pDS5 derivatives and directly subjected to translation. (B) Translation of mRNAs in the absence (lanes a, d, and f) or presence (lanes b, c, e, and g) of the respective 20-mer oligodeoxynucleotides. Arrows point at the translation products: prelysozyme (pLSM), truncated pLSM (pLSM/Sst and pLSM/Taq); invariant chain/CAT-fusion protein (Ii-CAT) and its truncated forms (Ii-2 and Ii-1); and CAT.

Procedure

Oligodeoxynucleotides. Synthetic oligodeoxynucleotides of 10, 15, and 20 bases were shown to efficiently arrest translation.[24] Routinely, we use 20-mers. Care should be taken not to choose a sequence for oligodeoxynucleotide hybridization which has homologies with more 5'-coding mRNA sequences. We observed that homologies between 12 and 15 out of 20 bases are sufficient to cause partial translation arrest.

The oligodeoxynucleotides can be used without extensive purification. The concentration at which a given oligodeoxynucleotide arrests translation has to be experimentally determined. In our hands, a 20-mer gives a complete block at a 50-fold molar excess of oligodeoxynucleotide over mRNA.

Translation Arrest in the Wheat Germ Cell-Free System. Transcription assay and translation premix are prepared as described above. For a 25-μl translation assay, 1 μl of appropriately diluted oligodeoxynucleotide (e.g., 0.25 μg/μl of a 20-mer), 16 μl of translation premix, and 3 μl of H_2O are added to 5 μl of transcription assay. The samples are incubated at 25° and processed for SDS–PAGE and autoradiography as described above.

Acknowledgments

[27] Use of Boronate-Containing Gels for Electrophoretic Analysis of Both Ends of RNA Molecules

By Gabor L. Igloi and Hans Kössel

The presence and location of one or more phosphate groups at the ends of RNA are characteristic of a particular RNA population. A knowledge of the precise state of terminal phosphorylation can supply information as to the function or to the pathways leading to the formation of a given RNA species (Table I).

In order to avoid traditional nucleolytic terminal analyses, which inevitably lead to the destruction of the starting material, we have developed