A coupled transcription-translation system from *Agrobacterium tumefaciens* and its application to study Ti plasmid expression in vitro

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ABSTRACT

A coupled transcription-translation system was isolated from *A.tumefaciens*. Expression of plasmids pBR322 and pKT212 from *E.coli*, cloned fragments of Ti plasmid (plasmids pSS155 and pSS156) and Ti plasmid derivatives pAL2803, pAL2811, pAL2821 and pAL2832 was analysed in an *A.tumefaciens* cell-free system and compared with their expression in an *E.coli* cell-free system. New proteins of 41K and 44K appeared in *A.tumefaciens* extracts, as the result of Ti plasmid expression. These proteins were not found in *E.coli* extracts.

INTRODUCTION

Agrobacterium tumefaciens induces the plant tumor, that is called crown gall, at wound sites on dicotyledons. A large plasmid (Ti plasmid) is essential for the tumor inducing capacity of *A.tumefaciens* (1). Part of the Ti plasmid is found to be integrated into the nuclear plant DNA of the tumor cell (1,2,3,4). On restriction endonuclease maps of Ti plasmids this part is indicated as T-region, whereas it is called T-DNA in the tumor cell. T-DNA specific transcripts are found in the transformed plant cells (5,6). Several functions, encoded by the plasmid DNA have been localized on Ti plasmid maps like octopine and nopaline catabolism and synthesis, conjugative transfer, origin of replication and virulence (7,8,9). Octopine and nopaline are arginine derivatives which are synthesized in crown gall cells and can be utilized only by *A.tumefaciens* (10). Generally, bacteria that induce octopine tumors catabolize octopine, whereas bacteria that induce nopaline tumors catabolize nopaline (1,9). Until now, little is known about expression into proteins of genes encoded by Ti plasmid DNA sequences in bacterial and plant cells (11, 12,13).

The cell-free coupled transcription-translation system of *E.coli* and protein synthesis in *E.coli* mini- and maxi-cells have proven to be very useful tools for analysing gene expression and have been used for characterization of several *E.coli* plasmids and their derivatives (14,15,16,17). However, when

we examined the expression of the Ti plasmid and its derivatives (18) in an *E. coli* cell-free system, we were unable to find any Ti plasmid specific protein. This could be due to a difference in the transcription-translation machinery between *E. coli* and *A. tumefaciens*. Therefore, we prepared a cell-free coupled transcription-translation system for both *A. tumefaciens* and *E. coli* to carry out a comparative study of the expression of Ti plasmid derivatives *in vitro*. The broad host range plasmid pKT212, that replicates in *Pseudomonas aeruginosa*, *E. coli* and *A. tumefaciens* (19,20), was used to find the optimum conditions of the coupled transcription-translation system of *A. tumefaciens*.

MATERIALS AND METHODS

Preparation of *A. tumefaciens* S-30 extract

A. tumefaciens strain C58, collection number LBA202 was used for preparation of a S-30 extract. Bacterial cells were grown in rich medium at 29°C (21). Cell growth was stopped at O.D.₅₅₀ = 0.7 by chilling with a frozen solution of 0.9% NaCl (200 ml per litre medium). Cells were collected by centrifugation at 8,000 rpm in a Sorvall SS-34 rotor for 15 min and washed with 10 mM Tris-HCl, pH 7.5, 10 mM MgOAc and 50 mM NH₄Cl. Cells were stored at -20°C until use.

The S-30 extract was prepared according to the procedure described for *E. coli* (22) with some modifications. Cells were disrupted with Alcoa and glass powder (3.5 g and 1 g, respectively per 1 g cells). The S-30 extract was incubated with DNase-Sepharose at 32°C for 30 min. The dialysis buffer contained in addition 3% polyethyleneglycol-6000.

Preparation of *E. coli* S-30 extract

E. coli strain KA81 (RNaseI⁻, phosphorylase⁻, met⁻, tyr⁻) was used. Cells were grown and S-30 extract was prepared according to Moorman *et al.* (22).

Coupled transcription-translation in *E. coli* extracts

The DNA-dependent transcription-translation system of *E. coli* we used, was as described by Zubay (23) with some modifications from Collins (14). A standard reaction mixture of 25 µl contained: 100 mM KOAc, 40 mM NH₄OAc, 50 mM Tris-HOAc, pH 8.0, 1 mM ATP, 0.25 mM GTP, UTP, CTP, 5 mM phosphoenolpyruvate, 20 µg/ml phosphoenolpyruvate kinase, 7 mM MgOAc, 0.05 mM each of 19 amino-acids, 1 µM methionine, 6 mM 2-mercaptoethanol, 30 µg/ml leucovarin Ca-salt (Lederle, Laboratories division U.S.A.), 2.6% polyethyleneglycol 6000, and 5 µl S-30 extract. Prior to the initiation of the reaction by adding 1-2 µg DNA

and 4-5 μCi ^{35}S -L-methionine (1000 Ci/mMole), the mixture was incubated for 15 min at 37°C. After incubation at 37°C for 20 min samples were treated according to Moorman *et al.* (22) and applied to 14-20% gradient or 15% polyacrylamide-SDS slab gels, according to Laemmli (24). Gels were run overnight at 4-8 mA and stained in 0.25% Coomassie brilliant blue in 50% methanol, 10% acetic acid in order to visualize the marker proteins (SDS-PAGE Standard Low Molecular Weight Markers, Bio-Rad: phosphorylase B-94K, bovine serum albumin-68K, ovalbumin-43K, carbonic anhydrase-30K, soybean trypsin inhibitor-21K, lysozyme-14.3K), destained in 15% methanol, 10% acetic acid, then treated with DMSO for 1 hour and impregnated with PPO (22.2% PPO in DMSO) for 3 hours, extensively washed with water, dried and prepared for autoradiography.

Coupled transcription-translation in *A. tumefaciens* extracts

The composition of the reaction mixture, optimum amount of DNA, ^{35}S -L-methionine and S-30 extract were similar to those for *E. coli* extracts except the concentration of KOAc and NH_4OAc , *i.e.* 60 mM and 20 mM, respectively and without addition of cold methionine. Protein synthesis was carried out for 20 min at 32°C without preincubation and samples were treated as described for *E. coli*.

Plasmid isolation

Plasmids pAL2803, pAL2811, pAL2821 and pAL2832 from *A. tumefaciens* were isolated according to Currier and Nester (25). Plasmids pBR322 and pKT212 from *E. coli* were isolated according to Clewell and Helinski (26). Plasmids pSS155 and pSS156 were a generous gift of Dr. S. Schweitzer (Max-Planck-Institut für Medizinische Forschung, Heidelberg).

RESULTS

Optimum conditions for A. tumefaciens cell-free system

The isolation procedure of the cell-free transcription-translation system of *A. tumefaciens* is essentially as described for *E. coli* (see Materials and Methods).

The fidelity of the *A. tumefaciens* cell-free system was determined using plasmids pKT212 (19) and pBR322.

Firstly, the extract was checked for DNase activity. More than 50% of the plasmid remained supercoiled after incubation with S-30 extract at standard conditions (data not shown).

Secondly, we determined the optimum temperature for protein synthesis (fig.

1a, lanes a-i), which appeared to be between 30°C and 32°C. This is in agreement with the optimum temperature for *A. tumefaciens* growth, *i.e.* 31.8°C (27). That we have isolated a coupled transcription-translation system of *A. tumefaciens* indeed, could be demonstrated by the action of rifampicin on plasmid DNA-directed protein synthesis. This antibiotic is an inhibitor of DNA-directed RNA synthesis in procaryotes. In our system rifampicin inhibited almost completely protein synthesis, as shown in fig. 1a, lane j.

Optimum salt concentrations were estimated for expression of both pKT212 and pBR322 in *A. tumefaciens* extracts (data not shown). The optimum conditions appeared to be 40 mM for KCl, 80 mM for KOAc, 40 mM for NH₄Cl and 80 mM for NH₄OAc. Fig. 1b shows the differences in expression of pKT212 in the *A. tumefaciens* S-30 extract, using different salt conditions. The combination of 60 mM KOAc and 20 mM NH₄OAc appeared to be most favourable for plasmid expression in *A. tumefaciens* extracts and was used in experiments described below.

Figure 1. Electrophoretic analysis in a 14-20% gradient polyacrylamide-SDS gel of protein synthesis directed by plasmid pKT212 in the *A. tumefaciens* cell-free system. (a) Synthesis at different temperatures, lanes: a-27°C, b-29°C, c-30°C, d-31°C, e-32°C, f-33°C, g-34°C, h-35°C, i-36°C. Lane j: synthesis at 31°C after the addition of rifampicin till 10 mg/ml. Lane k: synthesis at 31°C without plasmid. (b) Synthesis at different salt concentrations, lanes: a-40 mM KCl, b-80 mM KOAc, c-40 mM NH₄Cl, d-80 mM NH₄OAc, e-40 mM KOAc and 40 mM NH₄OAc, f-60 mM KOAc and 20 mM NH₄OAc, g-20 mM KOAc and 60 mM NH₄OAc. Lane h: synthesis at 60 mM KOAc and 20 mM NH₄OAc without plasmid.

Comparison of E.coli and A.tumefaciens systems

The specificity of *A.tumefaciens* and *E.coli* extracts was compared at optimum conditions for both systems.

Firstly, expression of plasmids pKT212 and pBR322 was investigated (fig. 2). The Ap^r- and Tc^r-gene *loci* of pBR322 gave products in both systems: a 34K band of the protein, responsible for tetracycline resistance (16) and 31K and 26K bands of β -lactamase (28). The expression products of pKT212 have not yet been identified, but the general protein pattern was the same in the two bacterial systems (fig. 2, lanes c,d). The overall rate of expression of both plasmids is lower in *A.tumefaciens*, but not for all genes. For example, expression of the Ap^r-gene *locus* was much less efficient in *A.tumefaciens* extracts (fig. 2, lane a), but the Tc^r-gene *locus* was expressed to the same extent in both systems (fig. 2, lane a). Inefficient expression of *E.coli* genes in *A.tumefaciens* extracts can not be explained by a difference in salt concentrations, because the inefficiency still remained when the same salt concentrations in both systems were used (data not shown). It might be explained by the difference in promoter affinity for *E.coli* and *A.tumefaciens* RNA polymerases or by poor translation in *A.tumefaciens* of messengers of *E.coli* origin.

Secondly, the expression of plasmids pAL2803, pAL2811, pAL2821 and pAL2832, with a molecular weight of 13.2 Mdal, 6.6 Mdal, 4.4 Mdal and 2.5 Mdal, respectively were analysed. These plasmids are derivatives of the octopine plasmid pTi-B6, which were constructed by B. Koekman in our laboratory (fig. 3) (18). All of them have the origin of replication of the Ti plasmid and the Ap^r-gene from transposon Tn1 as a selective marker. The latter gives rise in

Figure 2. Autoradiograms of 14-20% gradient polyacrylamide-SDS gels of proteins encoded by plasmids pBR322 (lanes a,b) and pKT212 (lanes c,d) and made in the cell-free systems of *A.tumefaciens* (lanes a,c) and *E.coli* (lanes b, d).

Figure 3. Linear map of the replicator region of pTi-B6, showing the DNA present in the various derivatives of this plasmid. Thick lines represent DNA derived from transposon Tn1. Only relevant restriction sites are shown, abbreviations used: *ori*, origin of replication; *stb*, plasmid stability and copy control; *inc*, plasmid incompatibility; *Ap^r*, ampicillin resistance, after Koekman *et al.* (18)

E. coli mini-cells to protein bands of 31K and 26K (29). In *E. coli* extracts these plasmids expressed only the *Ap^r*-gene of transposon Tn1: bands of 31K and 26K proteins (fig. 4, lanes a,b,c,h). In *A. tumefaciens* extracts at least one new protein of 41K appeared for pAL2803, pAL2811 and pAL2821 (fig. 4, lanes f,e,d) and one of 44K for pAL2832 next to a faint broad band corresponding to a protein of 41K (fig. 4, lane i). These data suggest, that for these plasmids an information encoded by Ti plasmid DNA sequences was recognized only in *A. tumefaciens* extracts.

The specificity of *A. tumefaciens* extracts for Ti plasmid DNA sequences was further analysed by studying the expression of the *EcoRI* fragment 7 from the octopine plasmid pTi-Ach5, cloned in both possible orientations in the *EcoRI* site of a pBR325 derivative (plasmids pSS155 and pSS156) (12). In *E. coli* extracts both plasmids gave a similar protein pattern, except for one protein of 15.5 K, which was shown earlier to be a fusion product, encoded partly by the vector and partly by the insertion (12). All other proteins of 28K, 27K, 25.5K, 24K, 23K, 21.5K and 21K can be encoded either by the vector part of the clones or by the Ti fragment (Fig. 5, lanes c,d). The pattern of proteins, synthesized in *A. tumefaciens* extracts was the same for both plasmids except for the fusion product, but differed from that observed for *E. coli*: protein bands of 28K, 25.5K and 21.5K were absent from the gel, when the products of pSS155 and pSS156 expression in *A. tumefaciens* extracts were analysed, whereas

Figure 4. Protein synthesis directed by plasmid pAL2821 (lanes a,d), plasmid pAL2811 (lanes b,e), plasmid pAL2803 (lanes c,f) and plasmid pAL2832 (lanes h,i) in the cell-free systems of *A. tumefaciens* (lanes d,e,f,i) and *E. coli* (lanes a,b,c,h). Lane g: synthesis in *E. coli* without plasmids. Proteins were analysed by electrophoresis in 14-20% gradient (lanes d,e,f,g,h,i) or 15% (lanes a,b,c) polyacrylamide-SDS gels.

bands of 26K and 23K were stronger and a band of 21K was weaker than found for *E. coli* extracts (fig. 5, lanes a,b).

DISCUSSION

Ti plasmids of *A. tumefaciens* are very large and have a coding capacity for more than hundred proteins. Up till now, no region of these plasmids has been clearly characterized by a gene-product relationship. Recently after *in vitro* translation of T-DNA specific mRNA's, isolated from plant tumor tissue, Mc Pherson *et al.* (11) have found two proteins. Schröder *et al.* (13) localized the structural gene for lysopine dehydrogenase on the octopine plasmid pTi-Ach5. Cloned fragments from the T-region of the octopine plasmid pTi-Ach5, when expressed in *E. coli* mini-cells, give several proteins but only in one orientation within the vector (12).

Figure 5. Protein synthesis directed by plasmid pSS155 (lanes a,c) and plasmid pSS156 (lanes b,c) in cell free systems of *A.tumefaciens* (lanes a,b) and *E. coli* (lanes c,d). Proteins were analysed by electrophoresis in a 14-20% gradient polyacrylamide gel.

We started our analysis of Ti plasmid DNA expression with the help of an *E. coli* cell-free system. However, the plasmids used could not reveal any Ti specific protein next to those encoded by the transposon Tn1. Therefore, we tried to isolate an *A.tumefaciens* cell-free system in order to have a homologous system for studying *A.tumefaciens* specific expression.

Specific *A.tumefaciens* expression was observed with Ti plasmid derivatives pAL2803, pAL2811, pAL2821 and pAL2832.

The extra 41K protein of pAL2803, pAL2811 and pAL2821 can be encoded by a region, located near the origin of replication of the octopine Ti plasmid, because it is encoded by all three plasmids in spite of their constructional rearrangements. Plasmid pAL2832 lacks a DNA fragment in the region near the origin of replication (fig. 3) and is probably therefore unstable in *A.tumefaciens* (18). This plasmid gave a new protein of 44K and a faint broad band of 41K. The observed difference in proteins might be somehow correlated with a difference in plasmid stability in *A.tumefaciens*. The absence of these proteins (41K and 44K) upon plasmid expression in *E.coli* extracts indicates, that their expression is not under the control of an *E.coli* promoter (the Ap^{r} -promoter of transposon Tn1).

Comparison of the expression of pSS155 and pSS156 in the *A.tumefaciens* and

E. coli cell-free systems revealed again that both systems differ significantly.

The difference we observed between the two bacterial systems can be on a transcriptional or translational level. *A. tumefaciens* Ti plasmids can be transferred to *E. coli* during conjugation, but the Ti replicon appears to be unable to maintain itself stably in *E. coli* (30). But even when the Ti plasmid is present in *E. coli* as a cointegrate with the Inc-P plasmid RP4, cells are unable to catabolize octopine, which was shown to be encoded by the Ti plasmid (30). Recent experiments have suggested differences in transcriptional activity between *E. coli* and *A. tumefaciens* RNA polymerases (S. Schweitzer, personal communication).

The *A. tumefaciens* cell-free coupled transcription-translation system described here seems to be the most suitable for *in vitro* analysis of the expression and organization of Ti plasmid genes.

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