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Expression of plant tumor-specific proteins in minicells of Escherichia coli: a fusion protein of lysopine dehydrogenase with chloramphenicol acetyltransferase

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ABSTRACT

Fragment EcoRI 7 from Ti-plasmid pTi Ach5, a part of the T-DNA in octopine tumors, was cloned in both orientations into pACYC184 and expressed in E.coli minicells. The cells synthesized four proteins from four different coding regions on EcoRI 7. Two of the proteins (M_r 25.000 and 26.000) were expressed with promoters from the Ti-plasmid fragment, while transcription for the two other proteins (M_r 18.000 and 74.000) started with a promoter on pACYC184. The M_r 18.000 protein represented a fusion product between chloramphenicol acetyltransferase (CAT) on pACYC184 and a part of lysopine dehydrogenase (LpDH), the enzyme synthesizing octopine and lysopine in plant tumor cells. The results suggest that E.coli minicells are a valuable system to study the proteins coded for by the T-region of Ti-plasmids.

INTRODUCTION

Crown gall, a neoplastic disease of many dicotyledonous plants, is the result of a unique system of naturally evolved genetic engineering (1,2). During infection of wounded plants a part of the Ti-plasmid, called T-region, is transferred from Agrobacterium tumefaciens into the nuclei of plant cells (3,4). This T-DNA is responsible for tumorous growth and synthesis of various substances, called opines, which can be used by the inducing bacteria as sole source of carbon, nitrogen, and energy (5).

The mechanism of this genetic colonization of plants is poorly understood. Transcripts from the T-DNA are found in nuclear and polysomal RNA (6-10), and at least some of the RNAs are translated in vitro into proteins (11, 12). The functions of these proteins in plant cells are unknown, with exception of the enzyme responsible for octopine synthesis (12).

It has proven difficult to identify tumor-specific proteins by translation of hybridization-selected mRNA since the concentration of T-DNA specific transcripts appears to be very low (12). The few proteins detected so far correspond to at most 20% of the coding capacity of the T-DNA, and since most

if not all of the T-DNA is transcribed (10) one can expect the existence of more T-DNA encoded proteins.

We are therefore developing a different approach to study T-DNA specific proteins which uses expression in E.coli to detect and define coding regions on Ti-plasmid fragments known to be present in transformed plant cells. Although proteins synthesized in the bacterial cells may not be identical in all aspects with their counterparts in plant cells, they can be expected to share amino acid sequences. These common antigenic sites could then be used to identify the rare T-DNA specific proteins in plants. We report here the first results obtained with this strategy: The detection of four coding regions on a specific Ti-plasmid fragment and the identification of the structural gene for lysopine dehydrogenase (LpDH), the enzyme responsible for octopine and lysopine synthesis in plant cells transformed with octopine Ti-plasmids.

MATERIALS AND METHODS

PLASMIDS AND BACTERIA

A bacterial strain containing fragment EcoRI 7 from octopine plasmid pTi Ach5 cloned in pBR325 was provided by the crown gall research group in Gent (Belgium) (38). Plasmids were isolated with the cleared lysate technique and two sequential centrifugations to equilibrium in CsCl (13). After digestion with EcoRI, fragments were extracted with phenol and precipitated with ethanol. For recloning the fragments were redissolved and ligated with pACYC184 (14) which had been linearized with EcoRI and treated with alkaline phosphatase. The products were used to transform E.coli 294, and colonies resistant to tetracycline (5µg/ml) and sensitive to chloramphenicol (100 µg/ml) and ampicillin (100 µg/ml) were selected. Of 100 colonies obtained, 85 showed the properties characteristic for EcoRI 7 inserts in pACYC184 (Tet^r, Cm^s,Ap^s), and 10 of these were further analyzed to obtain both orientations of the Ti-plasmid fragment in the plasmid vector. Transformation of the minicell producing strain E.coli DS410 (15) was carried out with the calcium technique (16). Restriction digests, alkaline phosphatase treatment, and fragment analysis were performed as recommended by the enzyme manufacturer (Boehringer, Mannheim, FRG). All cloning experiments were carried out under L1/B1 conditions as suggested and specified by the German Central Commission for Biological Safety (Berlin).

PROTEIN SYNTHESIS IN MINICELLS

Minicells of E.coli DS410 were isolated through 2-3 sucrose gradients (17) and stored in liquid nitrogen in M9 minimal medium containing 30% (v/v) glycerol. For protein synthesis, 1 A_{600} (10^{10} minicells) was suspended in 0.5 ml M9 medium containing 50 µM each of 19 amino acids except methionine and incubated for 10 min at 37°C. When cyclic AMP was used, it was added from a neutralized 0.1 M stock solution to a final concentration of 5 mM. Assays were mixed with 25 µCi [^{35}S]-methionine (800-1200 Ci/mmol, Amersham Buchler), and incubations were continued for 85 min at 25° or 37°C. Total incorporation of radioactivity was determined with 50 µl of the assay mixture. Proteins precipitated with trichloroacetic acid were dissolved and reduced by boiling in 50 mM Tris-HCl (pH 6.8), 2% dodecyl sulfate, 10% glycerol, and 1% 2-mercaptoethanol, and they were analyzed in slab gels (BioRad Laboratories) containing 12% polyacrylamide and 0.1% dodecyl sulfate (18). In some cases a 14-20% polyacrylamide gel was used. Radioactive proteins were visualized by fluorography (19) after treatment of the gels with Enhancer (New England Nuclear).

ANTISERA AND IMMUNOPRECIPITATIONS

Goat antiserum against chloramphenicol acetyltransferase Type I (CAT I) was provided by Prof. W.V. Shaw (Leicester, England), and rabbit antiserum against lysopine dehydrogenase purified from sterile plant tumor tissues was a gift from Prof. R.A. Schilperoort (Leiden, The Netherlands). Immunoprecipitations were performed with 0.1 ml of the assay mixtures. Pelleted minicells were resuspended in 78 µl 50 mM Tris-HCl (pH 8) and 12.5 µl lysozyme (10 mg/ml). After 5 min at room temperature, 10 µl of 0.25 M EDTA (pH 8) were added, and incubations were continued for 5 min. Minicells were then lysed by addition of 25 µl 0.25 M Tris-HCl (pH 7.5) containing 10% Triton X-100 and 0.75 M NaCl. After 30 min at 37°C, insoluble material was pelleted by centrifugation for 30 min at 22.000 x g at 4°C, and the supernatant fluids were incubated with 0.2 µl antiserum and 0.005% PMSF for 45 min at 37°C and then left overnight at 4°C. Proteins bound to antibodies were isolated with the Immunbead Second Antibody Immunoglobulin technique as recommended by the manufacturer (BioRad Laboratories).

MUTAGENESIS OF MULTICOPY PLASMIDS BY TRANSPOSON TN5

Tn5 mutagenesis of multicopy plasmids was carried out in a strain with a chromosomally located Tn5 transposon (E.coli C600, met::Tn5). Spontaneously occurring Tn5 transpositions onto multicopy plasmids were selected directly by using a gene dosage effect. Cells containing multicopy plasmids with Tn5 ininserts form large colonies on agar plates with enhanced neomycin concentra-

tion (150-500 µg/ml). To confirm plasmid located Tn5 transposons, colonies were lysed by lysozyme-SDS treatment (20). After phenol extraction the lysates were used for gel electrophoresis. Plasmids with Tn5 inserts band - as a result of their increase in size of approximately 6.000 bp - at a position different from that of the original plasmid.

MAPPING OF TN5 INSERTION SITES ON MULTICOPY PLASMIDS

Tn5 insertion sites of multicopy plasmids were determined by restriction analysis. Restriction sites within the inverted repeats of Tn5 (Xho I, Pst I, and Hind III) facilitate the analysis (21). Plasmid DNA was purified by a quick cleared lysate method using Triton X-100 as detergent. After phenol extraction and ethanol precipitation the DNA was directly used for restriction analysis. Digests were performed in presence of 1 mg RNAse/ml restriction buffer.

RESULTS

CLONING OF ECORI 7 IN PACYC184 AND EXPRESSION IN MINICELLS

Fig. 1A shows a restriction map of the T-region from the octopine plasmid Ach5 and the T-DNA present in several different plant tissues transformed with octopine Ti-plasmids (22). Fragment EcoRI 7 was chosen for expression in E.coli minicells since it covers a large part of the T-DNA, and genetical studies with Ti-plasmid mutants have shown that its right end contains part of the DNA responsible for octopine synthesis in tumors (23,24), suggesting a defined function for this region. Also, recent experiments have shown that the plant cells contain transcripts from this fragment (10).

EcoRI 7 was recloned from pBR325 into pACYC184 to facilitate detection of Ti-plasmid coded proteins. This vector expresses a chloramphenicol acetyltransferase (CAT I), and both protein and gene have been analyzed and sequenced (25-29). Plasmid pACYC184 is of special advantage since only CAT (M_r 25.000) is detected on polyacrylamide gels when the plasmid is expressed with the minicell technique. The single EcoRI site of pACYC184 is in the structural gene for CAT (14), and insertion of foreign DNA destroys chloramphenicol resistance, leaving a truncated gene which codes for a small residual protein (M_r 8.500). Thus, appearance of larger proteins in minicells incubations is likely to be the result of expression from coding regions of the Ti-plasmid fragment.

Fig. 1B describes the two orientations of EcoRI 7 in pACYC184 with respect to the position of the CAT promoter. The presence of both orientations

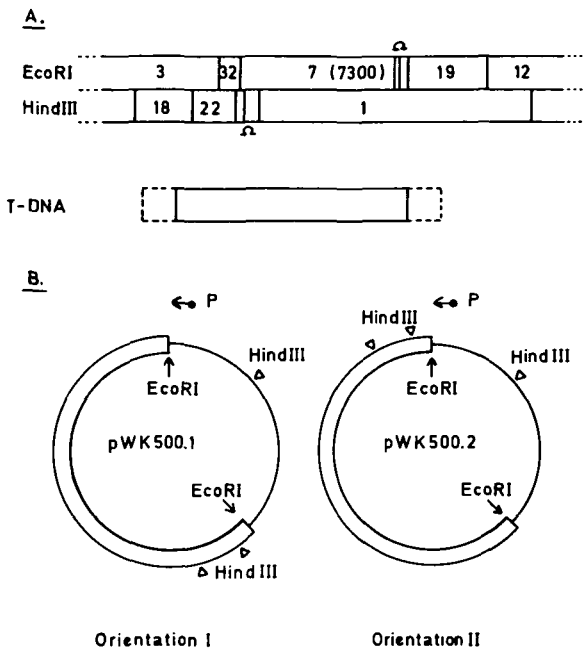

Fig. 1
A. Restriction map of the T-region in octopine plasmid Ach5, and T-DNA in various plant tissues transformed with the Ti-plasmid and expressing LpDH activity (22). The number in brackets indicates the base pairs in fragment EcoRI 7. The dotted lines in the T-DNA outline the limits of accuracy with which the boundaries of the T-DNA have been mapped (22).
B. Restriction maps of plasmids pWK500.1 and pWK500.2. Fragment EcoRI 7(double line) was cloned into the EcoRI site of pACYC184 (single line), resulting in two new plasmids containing EcoRI 7 in opposite orientations with respect to the CAT promoter (P): pWK500.1 = orientation I; pWK500.2 = orientation II. The two plasmids can be distinguished by digestions with Hind III which yield fragments of different sizes; pWK500.1: 7.700; 2.900 and 300 bp; pWK500.2: 8.800; 1.800 and 300 bp.

was confirmed by restriction analysis with Hind III which yields fragments of different size in the two orientations (data available on request). One plasmid was called pWK500.1 (orientation I), the other pWK500.2 (orientation II).

Plasmids pWK500.1 and pWK500.2 were used to transform the minicell producing strain E.coli DS410, and isolated minicells were incubated with radioactive methionine. Lane a and b of Fig. 2 show that they synthesized four major radioactive proteins larger than the residual protein encoded by the truncated CAT gene. Two of them (M_r 25.000 and 26.000) were found with both orientations of EcoRI 7, suggesting that the RNAs were transcribed from in-

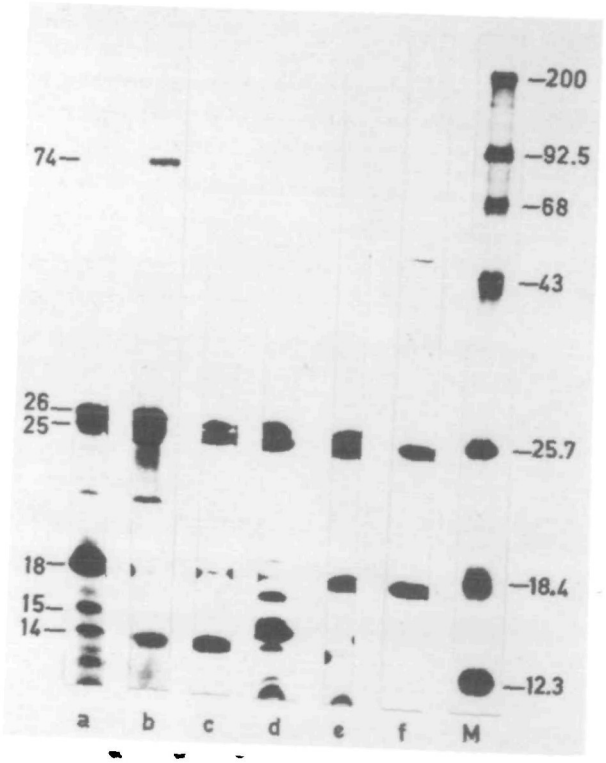

Fig. 2
Gel electrophoretic analysis of proteins synthesized in minicells. Lanes, a: pWK500.1; b: pWK500.2; lanes c-f: Protein patterns obtained with pWK500.1 which had been mutagenized with Tn5 in positions 1,2,4 and 14 (see Fig. 3); c: pWK500.1-1; d: pWK500.1-2; e: pWK500.1-4; f: pWK500.1-14. M: Marker proteins. The numbers indicate the size of the proteins in Daltons x 10^{-3}. Analysis was carried out on 14-20% polyacrylamide gels containing 0.1% dodecyl sulfate. Minicell incubations were performed at 25°C.

ternal promoters on the inserted DNA. The other two proteins were detected in one orientation only (I: M_r 18.000, lane a; II: M_r 74.000, lane b) which indicates that transcription started in the vector plasmid. These results suggested that at least four coding regions from EcoRI 7 were expressed in the minicells.

MAPPING OF THE M_r 18.000 PROTEIN BY TRANSPOSON MUTAGENESIS

To confirm that fragment EcoRI 7 in orientation I codes for the proteins observed in minicells, we mutagenized plasmid pWK500.1 with transposon Tn5 and analyzed the gene products of the cloned fragment. Tn5 mutagene-

sis was achieved by a method that makes use of the gene dosage effect resulting from the translocation of a chromosomally located Tn5 onto a multicopy plasmid. Cells containing pWK500.1::Tn5 can easily be detected on agar plates with enhanced neomycin concentration. Starting with an overnight culture of E.coli C600 met::Tn5 (pWK500.1) we could isolate strains with Tn5 inserted into plasmid pWK500.1. To map the Tn5 insertion sites, purified plasmid DNA was digested with different restriction enzymes, like XhoI and EcoRI, and the fragments were analyzed on agarose gels. Since the cloned fragment EcoRI 7 does not contain a restriction site for XhoI and since Tn5 is cut by XhoI at both ends in the inverted repeats, the Tn5 insertions of fragment EcoRI 7 can be mapped by simple calculations. A collection of such insertions is shown in Fig. 3.

The minicell technique was used to determine the effect on the protein patterns. Insertions 1 and 2 led to a reduction in the size of the M_r 18.000 protein (Fig. 2, lane c: M_r 14.000, pWK500.1-1; lane d: M_r 15.000 pWK500.1-2), indicating that the coding region was interrupted by Tn5. This effect was not observed with insertions 4 and 14 (lanes e and f, respectively) or with any of the other insertions (not shown). Tn5 in position 14 abolished formation of the M_r 26.000 protein, thus suggesting that this insertion affected the coding

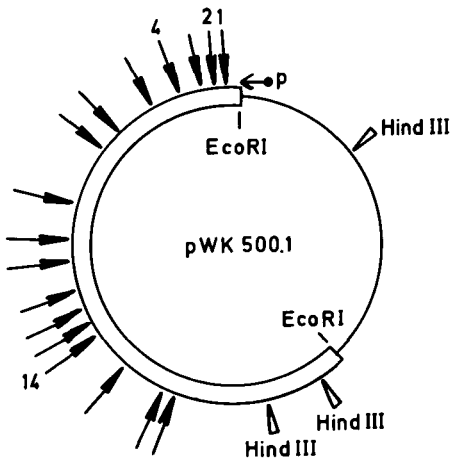

Fig. 3
Tn5 insertion sites in fragment EcoRI 7 of plasmid pWK500.1. Positions are indicated by arrows; they are numbered starting at the end near the CAT promoter (P). Tn5 mutants are identified by an affix; e.g. pWK500.1-1 is the plasmid with transposon Tn5 inserted in position 1. The sites were mapped with an accuracy of ± 50 bp.

region for this protein. The third, slightly smaller protein synthesized with pWK500.1 (M_r 25.000) could not be mapped since it comigrates with one of the Tn5 coded proteins.

The positions of insertions 1 and 2 together with the size of the corresponding proteins formed in minicells allow a fairly accurate determination of the region on pWK500.1 coding for the M_r 18.000 protein. Fig. 4 describes the results obtained assuming that three bp code for one amino acid and that the average mass of amino acids is 120. The coding region starts at about +220 bp in the vector plasmid pACYC184, overspans the EcoRI site and extends to about 230 bp into EcoRI 7. Thus, the M_r 18.000 protein appears to represent a fusion product containing the truncated CAT and part of a protein encoded on the Ti-plasmid fragment.

If this conclusion is correct, expression of the fusion protein is likely to be dependent on the CAT promoter whose position has been mapped (28,29). One would expect, then, that the rate of synthesis of the protein is stimulated by cyclic AMP, since it has been shown that expression of CAT is modulated to some extent by catabolite repression (30,31). While studying this effect we noticed that incubation at 37°C selectively favored expression of the fusion protein when compared with 25°C, and since our interest fo-

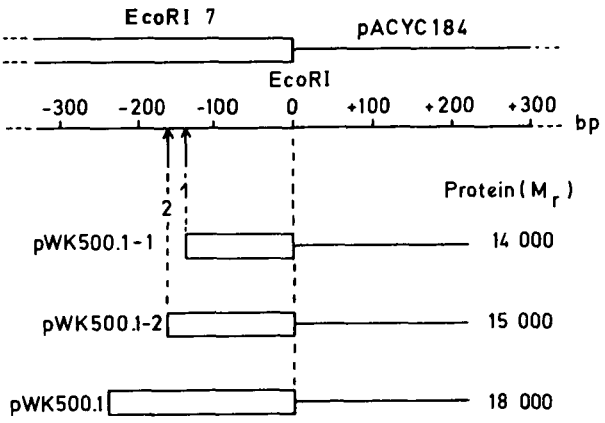

Fig. 4
Coding region for the M_r 18.000 protein synthesized with pWK500.1. The upper line shows the part of the restriction map of pWK500.1 which contains the EcoRI site near the CAT promoter (see Fig. 1). Tn5 insertions in position 1 and 2 (see Fig. 3) are indicated by arrows. The coding regions (lower part) were calculated from the positions of the Tn5 inserts and the size of the proteins synthesized with the mutagenized plasmids (Fig. 2).

cussed on this protein, all further experiments were carried out at the higher temperature. Fig. 5A (lanes a,b) shows the results of experiments which were performed in absence and presence of cyclic AMP. The results clearly suggest that the cyclic nucleotide stimulated formation of the M_r 18.000 protein. Direct measurements of immunoprecipitable radioactivity obtained with antiserum against CAT (see below) indicate that the increase amounted to 3-5 fold in independent incubations with different batches of minicells. Fig. 5, lane c also demonstrates that minicells containing no plasmids synthesized no significant amount of distinct proteins. All further experiments were performed in presence of cyclic AMP.

THE FUSION PROTEIN CONTAINS A PART OF LPDH

LpDH is an enzyme activity specific for octopine tumors (32-35). Experiments with Ti-plasmid mutants suggested that part of EcoRI 7 is necessary for

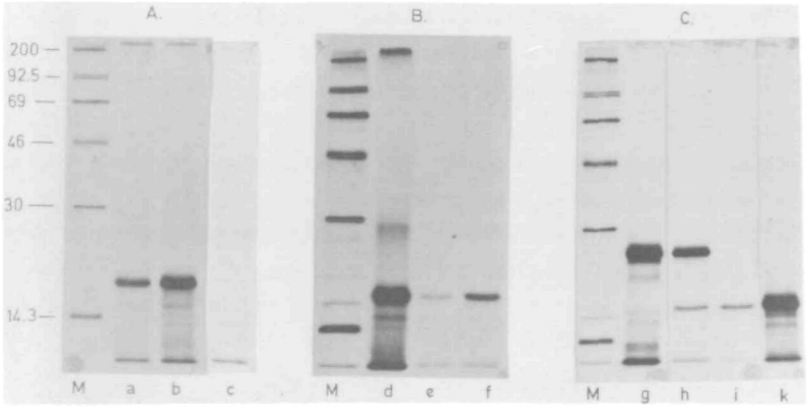

Fig. 5
Gel electrophoretic analysis of proteins synthesized with pWK500.1 in minicells. The gels contained 12% polyacrylamide and 0.1% dodecyl sulfate. M: Marker proteins; the numbers indicate their size in Daltons x 10^{-3}. Minicell incubations were performed at 37°C.
A. Effect of cyclic AMP on the expression of the M_r 18.000 protein. Lane a: Without cyclic AMP; b: +5 mM cyclic AMP; c: Minicells without plasmids (incubation with cyclic AMP).
B. Immunoprecipitation of the M_r 18.000 protein. Lane d: Total incorporation; e: Immunoprecipitate with CAT antiserum; f: Immunoprecipitate with LpDH antiserum.
C: Specifity of LpDH antiserum. Lane g: CAT, synthesized with vector plasmid pACYC184 in minicells; h: Immunoprecipitate obtained with CAT antiserum from a mixture of CAT and fusion protein; i: Immunoprecipitate obtained with LpDH antiserum from a mixture of CAT and fusion protein; k: Fusion protein, synthesized with pWK500.1 in minicells.

octopine synthesis in tumors (23,24), and a more detailed analysis has shown that the region of EcoRI 7 expressed in the fusion protein in minicells is part of the gene responsible for octopine synthesis (De Greve, H. et al., in preparation). If this gene is the structural gene for LpDH one would expect that the fusion protein is immunoprecipitated by antiserum against highly purified LpDH from plant tumor cells. Fig. 5B demonstrates that the fusion protein was recognized by LpDH antiserum (lane f) and also by antiserum against CAT (lane e). The following experiment was performed to exclude the possibility that the antiserum against LpDH reacted with CAT: Labelled minicell extracts containing either CAT synthesized with pACYC184 or fusion protein synthesized with pWK500.1 were mixed, and portions of the mixture were incubated with antiserum either against LpDH or against CAT. Analysis of the immunoprecipitates by gel electrophoresis showed that the CAT antiserum recognized CAT and the fusion protein (Fig. 5C, lane h), while the LpDH antiserum recognized only the fusion protein (lane i) although CAT was present in excess in the mixture. These findings demonstrate the specificity of the LpDH antiserum, they confirm the conclusion that the M_r 18.000 protein represents a fusion with CAT, and they identify the part encoded on the Ti-plasmid fragment EcoRI 7 as part of LpDH.

The question whether the two different antisera recognized the same protein was also investigated with another approach. If the M_r 18.000 protein is a true fusion between CAT and LpDH, one expects that the amount of radioactivity precipitated with the two antisera is comparable at different times during the minicell incubations. Fig. 6 shows that the values obtained with antiserum against either CAT or LpDH followed the same curve, indicating that the same protein was precipitated, and this was confirmed for all points by gel electrophoretic analysis (not shown). The results demonstrate furthermore a sharp drop of immunoprecipitable radioactivity after 1.5 h of incubation. This seems to reflect a high instability of the fusion protein in minicells.

None of the other proteins synthesized in minicells with either pWK500.1 or with pWK500.2 was immunoprecipitated with antiserum against CAT or LpDH. This is of special interest for the M_r 74.000 protein which was expressed only with orientation II of EcoRI 7 in pACYC184 (pWK500.2, see Fig. 2). The failure to precipitate with CAT antiserum suggests that this protein is not a fusion with CAT, but that its coding region is located entirely on the Ti-plasmid fragment, although transcription appears to start at the CAT promoter in pACYC184.

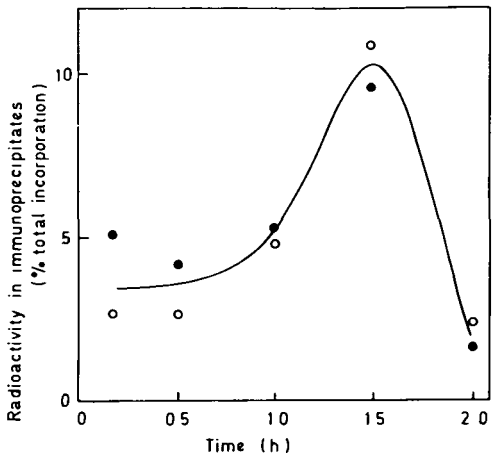

Fig. 6
Time course for the synthesis of immunoprecipitable protein formed with pWK500.1. The minicell incubations were supplemented with cyclic AMP (5 mM) at zero time. Portions of the assay mixtures were removed at the times indicated and analyzed for radioactivity immunoprecipitated with antiserum against CAT (o) or LpDH (•).

DISCUSSION

The T-region of Ti-plasmids is present both in Agrobacteria and in transformed plant cells and is transcribed into RNA both in the procaryotic (39) and in the eucaryotic cells (6-10). Within the T-region, fragment EcoRI 7 is of particular interest since most of it consists of sequences which are homologous in octopine and nopaline plasmids and which are essential for tumor induction in plants (40,41, and Engler, G. et al., submitted). Since these functions are characterized solely by the capacity of mutated plasmids to induce tumors, this operational definition does not distinguish whether they are expressed in the bacteria in the early stages of infection or whether they are required later after transfer into the plant cells. Interpretation of our results must therefore consider the possibility that EcoRI 7 may contain coding regions which are active in bacteria or plant cells, and there is no evidence which excludes that some coding regions are expressed both in procaryotic and eucaryotic cells.

The results show that fragment EcoRI 7 expresses four coding regions in minicells of E.coli, and Table 1 summarizes the data. In light of the possible dual role of some of these DNA sequences it is of special interest that two

Table 1

Expression of coding regions on Ti-plasmid fragment EcoRI 7 in minicells of E.coli

Size of protein (M_r)	Synthesized with plasmid pWK500.1	Synthesized with plasmid pWK500.2	Position mapped by Tn5 mutagenesis	Comments
18.000	+	-	yes (pWK500.1-1) (pWK500.1-2)	Transcription from CAT promoter on pACYC184; fusion protein from truncated CAT and carboxy-terminal part of LpDH; very strong expression
25.000	+	+	no	Transcription from promoter on EcoRI 7; strong expression
26.000	+	+	yes (pWK500.1-14)	Transcription from promoter on EcoRI 7; strong expression
74.000	-	+	no	Transcription from CAT promoter on pACYC184; but protein encoded entirely on EcoRI 7; weak expression

proteins (M_r 25.000 and 26.000) are expressed from internal promoters, suggesting that the fragment contains transcriptional and translational signals which can be used in a procaryotic background. Since the T-region is transcribed in the Agrobacteria (39) it seems possible that these coding regions are expressed in the Agrobacteria during tumor induction. However, the transcription studies did not define single coding regions, and it is not known whether transcription uses the same signals in Agrobacteria and in E.coli. Also, expression in E.coli minicells does not always reflect the situation in maxicells (see Refs. 42-44 for detailed discussions), and therefore this possibility must be considered with caution without further evidence.

With three of the four coding regions we do not know at present whether they are also expressed into protein in plant cells. T-DNA specific proteins with the size of those formed in minicells (M_r 25.000, 26.000, 74.000) were not detected so far after translation in vitro of plant RNA selected by hybridization to EcoRI 7 (Schröder, G. et al., unpublished), but this may be due to the very low rate of expression which hampers all experiments with this approach (12). However, the more sensitive method of Northern-Blotting showed recently that EcoRI 7 hybridized to four distinct RNAs from tumor cells (Willmitzer, L. et al., unpublished). Interestingly, the four regions coding for plant transcripts appear to correlate with the four regions expressed in E. coli minicells, as far as they are defined at present. Experiments to be published elsewhere suggest that the plant transcripts are larger than the regions expressed in minicells, and in all cases investigated the regions expressed in minicells appear to lie within the regions transcribed in plant cells. Although other explanations are not ruled out, it is tempting to speculate that plant cells and E.coli minicells express at least partly the same coding regions. According to our strategy, antibodies raised against the proteins formed in E.coli minicells will present a powerful tool to investigate these questions.

The feasibility of this approach is demonstrated by the detection of LpDH-specific amino acid sequences expressed in one of the proteins formed in bacterial minicells. Our results indicate that part of the structural gene for this plant tumor-specific protein is on restriction fragment EcoRI 7 of the octopine plasmid and that this part is expressed in minicells as fusion protein with CAT. This conclusion is based on the following observations:

(i) the protein is expressed in only one orientation (Fig. 2),
(ii) its synthesis is modulated by cyclic AMP (Fig. 5A),
(III) the coding region was mapped by a combined method using Tn5 mutagenesis

and expression in minicells (Fig. 2 and 3). This method was developed by one of us (W. Klipp) and was first employed to study the coding regions for nitrogen fixation in Klebsiella (36),

(iv) the protein was specifically recognized by antiserum against CAT and by antiserum against LpDH which had been purified from sterile octopine tumor cultures (Fig. 5B, C).

LpDH is a M_r 39.000 ± 1000 protein in plant cells (34,35,37). Since the fusion protein (M_r 18.000) contains the residual CAT (M_r 8.500) (26,27), the remainder of M_r 9.500 represents LpDH specific amino acid sequences. This corresponds to about 25% of the total enzyme, and the coding region is at the right end of EcoRI 7 as drawn in the restriction map of the T-region in Fig.1A. Transcription proceeds from the right to the left, and if the gene contains no introns, the part expressed in E.coli represents the carboxy-terminal end of the enzyme. Accordingly, about 75% of the gene should be to the right of EcoRI 7, spanning the two omega fragments and extending partly into EcoRI 19. This is consistent with the recent finding that the omega fragments can be used to select from plant RNA the mRNA for LpDH (Schröder, G. et al., unpublished). This model places the start of the gene within the known boundaries of the T-DNA, suggesting that not only part, but all of LpDH is encoded on the Ti-plasmid. This conclusion is consistent with the finding that this region of the Ti-plasmid contains an intron-free open reading frame which could account for a protein of the size of LpDH (De Greve, H. et al., in preparation). Also the fact that LpDHs from various independently transformed tumor lines are very similar in size and other properties (34,35,37) argues that the complete structural gene for this enzyme is on the Ti-plasmid. This supports the hypothesis that genetic manipulation of plants by Agrobacteria involves transfer of the structural gene for LpDH into plant cells.

Our results suggest that expression in E.coli minicells is useful to analyze coding regions in the T-region of Ti-plasmids. With respect to a general application of this approach to other DNAs of unknown functions it is necessary to note some points: The T-region of Ti-plasmids may be more amenable to this type of analysis than DNA from a eucaryotic source, since some of the coding regions may be expressed in the procaryotic Agrobacteria. With DNA occurring only in eucaryotic cells it may be necessary to aim for a fusion with a bacterial gene, as described here for LpDH. Although this requires matching reading frames, this should be possible in many cases by cloning overlapping restriction fragments into vectors which allow joining to bacterial genes in different reading frames.

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