THE mRNA FOR LYSOPINE DEHYDROGENASE IN PLANT TUMOR CELLS IS COMPLEMENTARY TO A Ti-PLASMID FRAGMENT

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1. Introduction

The interaction between the soil bacterium *Agrobacterium tumefaciens* and many dicotyledonous plants represents a unique system of naturally evolved genetic engineering [1,2]. During infection of wounded plants a part of the Ti-plasmid, called T-DNA, is transferred from the bacteria into the nuclei of the plant cells, and is responsible for the known phenotypes of crown gall cells: Tumorous growth and synthesis of new substances, called opines, which are used specifically by the bacteria as sources of carbon, nitrogen, and energy. Studies with tumor inducing mutants of Ti-plasmids suggest that opine synthesis is controlled by a specific region on the T-DNA [3,4]. However, such experiments cannot resolve the question whether the T-DNA operates by activation of otherwise not expressed plant genes or whether T-DNA itself contains structural genes which are active in eucaryotic cells. We present results suggesting that the octopine plasmid pTi Ach5 codes for lysopine dehydrogenase, the enzyme responsible for octopine and lysopine synthesis in transformed plant tissues [5,6].

2. Experimental

Tobacco crown gall line A6-S1 harbours the T-region of octopine plasmid pTi A6 and expresses lysopine dehydrogenase activity. The tissue used as control, line NW-S1, is a normal tobacco culture free of T-DNA and without lysopine dehydrogenase activity, but it is independent of hormone-addition as are cells transformed by Ti-plasmids [1,2]. Both cultures were grown in suspension in hormone-free medium [7].

Hybridization-selection of T-DNA specific mRNA from plant cells was performed with two different fragments of octopine plasmid pTi Ach5 cloned in pBR 322 (fig.1): Clone pGV 0153 (*Bam*HI 8; 7.3 kilobases) and pGV 0201 (*Hin*dIII 1; 12.6 kilobases). Mapping of the T-DNA in the tobacco line A6-S1 and cloning of the Ti-plasmid fragments will be described elsewhere (De Beuckeleer, M. et al., in preparation). The map is consistent with published data [8]. Digested plasmid DNA was coupled to diazotized microcrystalline cellulose as in [9].

Hybridizations of plant RNA to bound Ti-plasmid fragments were performed with polysomal RNA [10] under R-loop conditions [11]. After removal of non-bound RNA by 6 washes for 10 min with hybridization buffer, hybridized RNA was eluted [11], precipitated 3 times from ethanol, and translated in a cell-free system prepared from wheat germ as in [10].

For immunoprecipitations, cell-free incubations were adjusted to 0.05 M Tris–HCl (pH 7.5), 0.15 M NaCl, 2% Triton X-100 (wash buffer) and mixed with 1 μl antiserum against lysopine dehydrogenase. After 1 h at 37°C samples were left overnight at 4°C. Proteins bound to antibodies were recovered with protein A coupled to Sepharose IVB (Pharmacia) according to recommendations of the manufacturer. Immunoprecipitates were washed 3 times with wash-buffer before elution with electrophoresis buffer containing 2% dodecyl sulfate [10].

Radioactive proteins were analyzed in 12% polyacrylamide gels containing 0.1% dodecyl sulfate [12] and visualized by autoradiography after treatment of gels with Enhancer (New England Nuclear). Purification of lysopine dehydrogenase and raising of specific antiserum will be described elsewhere (H. H., et al., in preparation). The purified unlabelled enzyme was analyzed by gel electrophoresis in presence of dodecyl sulfate in a slightly modified system [12,13].

3. Results and discussion

If the structural gene for lysopine dehydrogenase is on the Ti-plasmid, mRNA for the enzyme should be complementary to the T-region of the Ti-plasmid. This should not be the case if T-DNA operates by activation of a plant-specific gene, since Ti-plasmid DNA does not hybridize to DNA of normal plant cells ([8,14] De Beuckeleer, M. et al., in preparation). We investigated therefore, whether or not transformed plant cells contain mRNA which can be selected by hybridization to the T-region of the Ti-plasmid and which, after translation in vitro, yields a protein identified as lysopine dehydrogenase by size and by specific immunoprecipitation. Hybridizations were performed with 2 cloned fragments from the T-region of octopine plasmid pTi Ach5 which together cover the entire T-DNA in the transformed tobacco line A6-S1 (fig.1). Experiments with RNA from a normal tobacco line (NW-S1) were included as controls for the specificity of the mRNA selection and of the immunoprecipitation.

Fig.2 shows the analysis of proteins synthesized in vitro with RNA from transformed and normal cells which had been hybridized to the Ti-plasmid fragments. The autoradiography revealed in all lanes a number of radioactive proteins which were also present in the control incubation of wheat germ extract without added RNA (a), indicating that they represent mRNAs endogenous to the wheat germ. RNA from transformed cells and selected by hybridization to clone 0201, however, produced an additional protein with the size of lysopine dehydrogenase (M_r 39 000, (b)). Such a distinct protein was not detected in control experiments with RNA from normal tissue (d,e) and with RNA from transformed cells hybridized to clone 0153 (c). Immunoprecipitations with antiserum against lysopine dehydrogenase show that the additional protein was recognized by the antibodies (fig.3(b) and that the corresponding control with RNA from normal tissue did not produce this protein (c). The size of the precipitated protein corresponded to that of purified lysopine dehydrogenase (fig.3(d)).

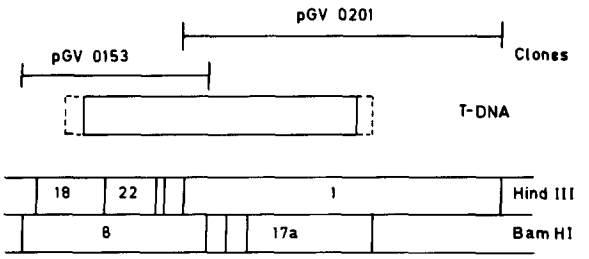

Fig.1. Ti-plasmid fragments used for selection of T-DNA specific mRNA from plant cells (pGV 0153 = *Bam*HI 8; pGV 0201 = *Hind*III 1), T-DNA in transformed tobacco cell culture A6-S1, and restriction map of the T-region in octopine plasmid pTi Ach5. The dotted lines in the T-DNA indicate that the precise borders are not known.

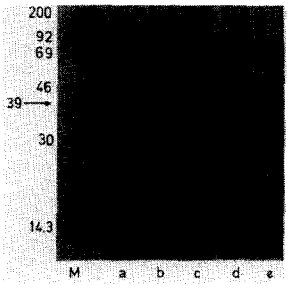

Fig.2. Gel electrophoretic analysis of total radioactive proteins synthesized in vitro with hybridized RNA: (a) Wheat germ extract without added RNA; (b) RNA from transformed cells bound to clone pGV 0201; (c) RNA from transformed cells bound to clone pGV 0153; (d) RNA from normal tissue bound to clone pGV 0201; (e) RNA from normal tissue bound to clone pGV 0153. (M) Marker proteins, the numbers indicate their size in $M_r \times 10^{-3}$; myosin, M_r 200 000; phosphorylase B, M_r 92 000; bovine serum albumin, M_r 69 000; ovalbumin, M_r 46 000; carbonic anhydrase, M_r 30 000; lysozyme, M_r 14 300. The arrow indicates the position of the tumor-specific protein (M_r 39 000) synthesized with RNA from transformed cells hybridized to clone pGV 0201.

The concentration of mRNA for the tumor-specific protein appeared to be very low. Our experiments suggest that it represented ~0.0001% of the total mRNA activity in transformed plant cells. Several lines of independent evidence indicate that this value is not an underestimate due to excessive losses during the experiments. Hybridization studies showed that transcripts from the total T-DNA represent ~0.0004% of poly(A)-containing RNA (Willmitzer, L. et al., submitted), and pulse-labelling of the cells, followed by partial purification and immunoprecipitation, led to similarly low levels for the rate of enzyme synthesis in vivo (J. S., et al., unpublished). The same experiments as described here were performed with another independently transformed tobacco culture (B6S3-S1) which harbours the T-region of octopine plasmid pTi B6S3. The results were essentially the same, except that the concentration of mRNA was even lower than

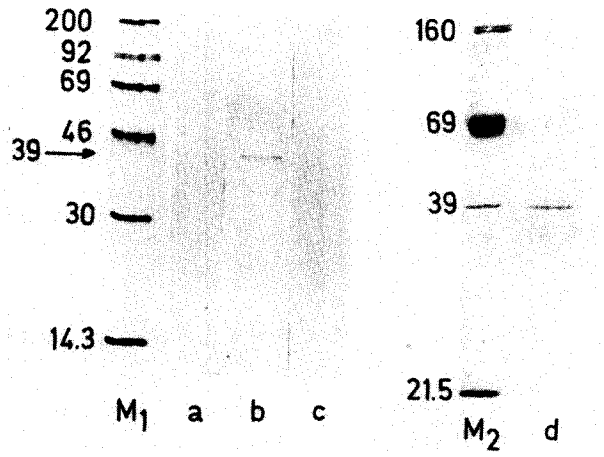

Fig.3. Analysis of immunoprecipitates and purified lysopine dehydrogenase: (a–c) Immunoprecipitates obtained with antiserum against lysopine dehydrogenase after translation of hybridized RNA in vitro. (a) Wheat germ extract without added RNA; (b) RNA from transformed cells annealed to clone pGV 0201; (c) RNA from normal tissue annealed to clone pGV 0201. (M_1) Marker proteins (see legend to fig.2). Unlabelled, purified lysopine dehydrogenase (d) was analyzed in a separate experiment in a different system (see section 2) and with different marker proteins (M_2): RNA polymerase subunits β and β', M_r 165 000 and 155 000; bovine serum albumin, M_r 69 000; RNA polymerase subunit α, M_r 39 000; trypsin inhibitor, M_r 21 500.

in A6-S1. All of these data suggest that this tumor-specific protein is coded by very low abundant mRNA [15]. This may be the reason for a previous failure to detect the mRNA for lysopine dehydrogenase [16].

Our experiments suggest that the structural gene for lysopine dehydrogenase resides on the Ti-plasmid. This conclusion is based on the observation that a specific fragment from the T-region of the octopine plasmid selects from transformed cells a mRNA coding for a protein which has the correct size and which is immunoprecipitable with a specific antiserum directed against highly purified lysopine dehydrogenase. The specificity of the approach was demonstrated by the fact that experiments with RNA from normal cells or with RNA from transformed cells hybridized to a different Ti-plasmid fragment did not yield a detectable signal. Localisation of the gene on fragment *Hin*dIII 1 (clone 0201, fig.1) is consistent with genetic evidence that the right end of the T-DNA is necessary for octopine synthesis in plant cells [4,17].

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