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Cloning of double stranded DNAs derived from polysomal mRNA of maize endosperm: isolation and characterisation of zein clones

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ABSTRACT

Starting from polysomal RNA of developing maize endosperm and applying the cloning procedure of integrating dsDNA into the Pst I site of plasmid pBR 322, clones containing sequences complementary to endosperm mRNAs were obtained. 25 per cent of these clones were identified as containing zein specific DNA sequences which hybridized either with the zein mRNA coding for the 22 000 M_r protein or with the zein mRNA coding for the 19 000 M_r protein. The zein-specific DNA inserts of the recombinant plasmids were further characterized by restriction enzyme analysis.

INTRODUCTION

The endosperm of developing maize kernels has proven to be a suitable system for the study of differentiation in plant tissues (1). At defined stages of development specific functions are expressed, some of them such as the storage proteins zein in high dose and with high tissue specificity. So far the molecular analysis of these processes has been performed mainly at the level of proteins (2). With the exception of the mRNAs coding for zein proteins, no other mRNA from endosperm has yet been isolated (3,4,5). In an effort to establish the basis for a more detailed analysis of nucleic acids involved in endosperm development, we have adopted the new cloning technology for a cloning of mRNA sequences related to endosperm functions. Starting from total polysomal RNA of developing endosperm, ds cDNA was synthesized and integrated into the Pst I site of plasmid pBR 322 (6) by means of the G/C connector method followed by transformation of E.coli C 600 $^-$ cells. About 25 per cent of the endosperm specific clones obtained were then identified with the help of biologically active zein mRNA as clones containing DNA sequences coding for zein protein. The zein-specific DNA inserts have relatively few restriction enzyme sites. Zein clones have recently also been constructed by the A/T connector method, however, not by starting from total polysomal

RNA but from purified zein mRNA isolated from protein bodies (7).

MATERIALS AND METHODS

REAGENTS: All reagents used were as previously described (5,8).

CONSTRUCTION OF RECOMBINANT DNA PLASMIDS: Poly(rA)-containing polysomal RNA from 21 days postpollination maize endosperm isolated as described previously (5) with an additional CsCl purification step according to Glisin et al (9) was used as template for cDNA synthesis with reverse transcriptase from AMV virus (provided by Dr. Beard through the Office of Program Resources and Logistics, Virus Cancer Program, National Cancer Institute). A 1.5 ml reaction mixture contained 50 mM Tris/HCl, pH 8.2, 10 mM $MgCl_2$, 100 mM KCl, 16 mM ß-mercaptoethanol, 1.2 mM each of dATP, dTTP and dGTP, 0.45 mM dCTP, 0.6 A_{260} oligo $(dT)_{12-18}$, 4 A_{260} units of mRNA and 123 units of enzyme. After incubation for 90 minutes at 38°C followed by alkali treatment (0.25 N, 18 hrs, 37°C), phenolisation and Sephadex G-50 chromatography 30 µg cDNA was obtained.

15 µg cDNA was converted to dsDNA by incubating it for 2 hours at 30°C in 1 ml reaction mixture containing 0.11 M K-phosphate pH 6.9,9 mM $MgCl_2$, 9 mM dithioerythritol, 0.36 mM each of the four deoxyribonucleoside triphosphates and 70 units of DNA polymerase I (Klenow form; from Boehringer, Germany). After phenolisation and Sephadex G-50 chromatography, 30 µg dsDNA was recovered. In order to remove the hairpin-structure of the dsDNA, 15 µg of dsDNA was incubated for 30 min at 30°C with 4 units of S_1 nuclease (prepared according to Vogt (10) (one unit digests 13 µg fd DNA to trichloracetic acid soluble material in 10 minutes at 45°C) in a solution of 210 µl containing 0.37 mM Na-acetate pH 4.6, 0.37 M NaCl and 5 mM $ZnSO_4$. After adding EDTA (to 20 mM) and SDS (to 0.6 %) and after phenolisation and Sephadex G-50 chromatography, the dsDNA was tailed at its 3' ends with a $(dC)_n$ sequence with the help of terminal transferase (prepared according to Yoneda et al (11)). An incubation mixture of 80 µl containing 0.1 M Na-cacodylate, pH 6.9, 1 mM $CoCl_2$, 0.05 mM dithioerythritol, 1,25 mM dCTP, 1.6 ug of dsDNA and 1 ug of terminal transferase was incubated for 10 minutes at 30°C and then terminated by heating for 2 minutes at 45°C. Likewise dG residues were added to the 3' ends of pBR 322 DNA (purified according to Sharp et al (12) with an additional sucrose gradient step (5 % - 20 %) and linearized with restriction enzyme Pst I) by incubating 10 ug DNA with 1.5 ug terminal transferase and replacing dCTP for dGTP in

an assay and under conditions as described for the dsDNA. For the subsequent annealing reaction, portions from the terminal transferase reactions, corresponding to 0.5 µg plasmid DNA and 0.04 µg dsDNA were added to 500 µl of 10 mM Tris/HCl, pH 8.0, 100 mM NaCl and 10 mM EDTA, heated for one hour at 60°C and for further 12 hours at slowly falling temperatures between 60°C and 40°C. After addition of 50 µg E.coli t-RNA and ethanol precipitation the nucleic acids were redissolved in 50 µl 10 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$ and 10 mM $CaCl_2$.

TRANSFORMATION AND IDENTIFICATION OF RECOMBINANT CLONES:

The annealed nucleic acids contained in the 50 ul buffer mix were added to 1-2x10^9 cells of $CaCl_2$ treated E.coli C 600$^-$ prepared according to Berg et al (13) suspended in 200 µl 0.1 M $CaCl_2$. After incubation for 30 minutes at 0°C and for 2 minutes at 42°C the bacteria were added to 2 ml nutrient broth containing tetracycline (1 µg/ml), incubated for 2 hours at 37°C and then, after distributing the 2 ml onto 6 agar plates (1.5 % agar) containing tetracycline (10 µg/ml), further incubated for 48 hours at 37°C. The obtained colonies were tested for ampicillin sensitivity by replica plating them on agar plates containing ampicillin (30 µg/ml). The analysis for tetracycline resistant and ampicillin sensitive colonies was repeated once leading finally to 35 colonies. The colonies obtained from II parallel experiments were together submitted to the hybridisation test.

COLONY HYBRIDISATION OF RECOMBINANT PLASMIDS:

The colonies received by the antibiotic screening were grown on nitrocellulose filters and submitted to colony hybridisation according to the procedure of Grunstein and Hogness (14). The hybridisation of the fiters with ^{125}Iodine-labelled RNA was performed for 24 hours at 37°C in a solution containing 5xSSC and 50 % formamide. For reaction with ^{125}I labelled cDNA the filters were incubated for 36 hours at 63°C as described by Mears et al., (15). The labelling of the RNA and DNA with ^{125}Iodine was done as described by Prensky (16). Autoradiography was performed with Kodak X-Omat films at - 70°C.

IDENTIFICATION OF ZEIN CLONES BY IN VITRO TRANSLATION:

The DNA of recombinant plasmids was isolated as described by Sharp et al.,

(12) from liquid cultures of individual clones grown in the presence of tetracycline (2 µg/ml). 10 µg of the purified DNA was then hybridized under R-loop conditions with 5 µg of the poly (rA) containing polysomal mRNA from endosperm followed by bio-gel chromatography of the hybridisation mixture according to the procedure of Woolford and Rosbash (17). The hybridized material isolated by ethanol precipitation from the void volume of the bio-gel column was heat denatured and tested for mRNA activity by in vitro translation in a wheat germ system. The conditions for the incubation assay as well as for the product analysis by polyacrylamide electrophoresis are as described recently (5).

SAFETY CONSIDERATIONS:

Cloning experiments and growth of recombinant plasmids were performed under L2/B I conditions as specified by "Richtlinien zum Schutz vor Gefahren durch in vitro rekombinierte Nukleinsäuren" of the BMFT of the Federal Republic of Germany.

RESULTS AND DISCUSSION:

As the cloning procedure described in this communication has recently been used for the cloning of DNA sequences derived from different mRNAs (18, 19), only some major points and modifications of the method are described.
The RNA employed as starting material for the cDNA synthesis was isolated from polysomes of 21 days post-pollination endosperms and represents a population of poly(rA)-containing mRNAs active in translation at this time in development. A major proportion of this mRNA consists of the mRNAs coding for zein proteins thus leading at the later transformation step to a considerable percentage of zein specific clones. The rRNA which still represents about 75 per cent of the polysomal mRNA preparation did not interfere with the synthesis of cDNA. The cDNA synthesis was optimized on an analytical scale and resulted with 30 % yield in a product of relatively uniform size of 10 - 12 S if analyzed by alkaline sucrose gradients. The conversion of the obtained cDNA to ds cDNA was selfpriming, however, the incubation temperature had to be kept below 32°C. For the connection of ds cDNA with pBR 322 the G/C homopolymer combination was chosen as, in this case, Pst I restriction enzyme sites, at which the plasmid had previously been linearized, are regenerated at the ends of the insert thus allowing a precise excising of the

DNA insert. Each plasmid preparation was purified in a sucrose gradient before proceeding with the enzymatic reactions. The transformation was carried out with E.coli C 600^{-} cells. The transformation efficiency with pBR 322 supercoil-DNA was 1-5x10^{6} tetracycline-resistant colonies per µg of DNA. Transformation assays with annealing mixtures consisting of $(dC)_n$-tailed dsDNA and of $(dG)_n$-tailed pBR 322 were first screened for tetracycline resistance and then for ampicillin sensitivity. In order to reduce the number of unstable transformants the screening with the antibiotics was repeated once, yielding finally about 400 colonies. These colonies were then submitted to a colony hybridisation analysis (14) with ^{125}Iodine-labelled cDNA. As can be seen from figure I, about 100 colonies contain recombinant plasmids with an endosperm specific DNA insert long enough to be detectable under these conditions. For a further identification of these endosperm-specific clones, reasonably pure RNA species from the developing endosperm are needed. At the time of this investigation, however, only zein coding mRNAs were available so that the obtained endosperm clones were screened for clones containing zein-specific DNA sequences.

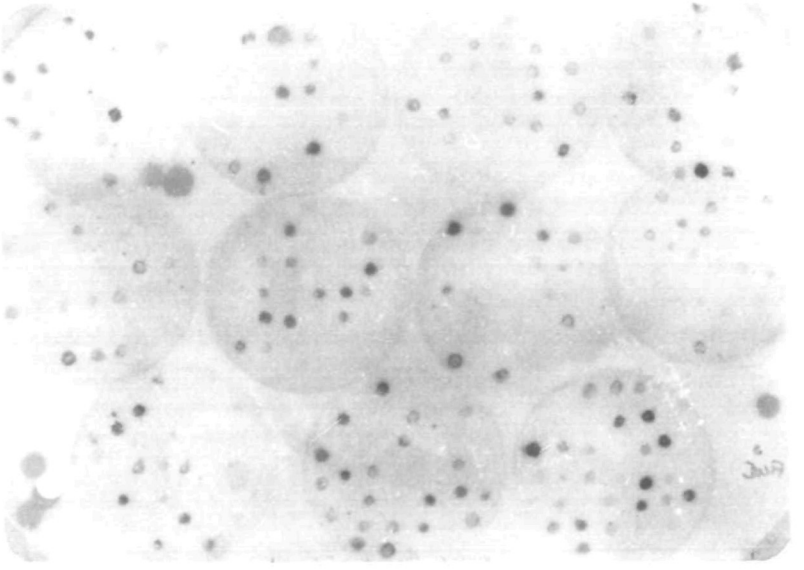

Figure 1: Autoradiography of colony hybridisation test with cDNA
11 nitrocellulose filters (diameter 4.5 cm) each carrying about 40 colonies were submitted to colony hybridisation as described in Methods. The hybridisation of all filters was performed in a total volume of 10 ml hybridisation solution containing 1.5 ug of ^{125}Iodine labelled cDNA (5x10^{6} cpm/ug). The photography shows the autoradiography of all II filters

This was done by a colony hybridisation analysis using as hybridisation probe a fraction of endosperm mRNAs, enriched for zein mRNAs (5). This in situ hybridisation yielded 25 colonies which were then analyzed in a preliminary fashion for the appropriate size of the DNA insert in the plasmids and for the presence of intact Pst I sites at the ends of the inserted DNAs. The result was that the DNA inserts were from 200 - 800 base pairs long and that the inserts could be excised by the Pst I enzyme from 22 plasmids. Two of these plasmids with relatively long inserts and with Pst I sites flanking the DNA inserts were then selected for a detailed analysis. The highly purified DNAs of these plasmids (pFW 13 and pFW 19) were submitted to a hybridisation reaction under R-loop conditions with polysomal mRNA from endosperm. The hybrid structures were then isolated by gel filtration and after denaturation analyzed for specific mRNA activity (17). Figure 2 shows the result of a re-

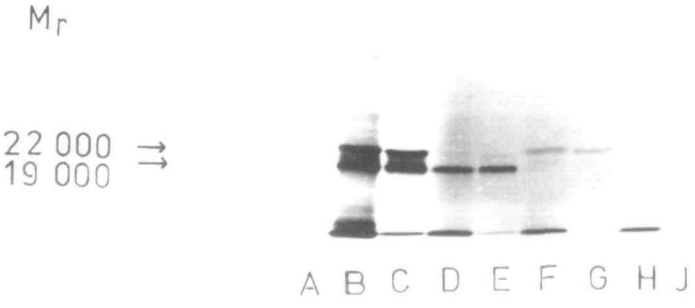

Figure 2. Polyacrylamide gel electrophoresis of proteins synthesized in vitro 50 ul standard wheat germ incubation assays according to Wienand et al., (5) with mRNA as specified below were incubated at 29°C for one hour. The proteins synthesized in 45 µl incubation mixtures were analyzed by polyacrylamide gel electrophoresis as described recently (5). The incubation used for slot A was synthesized without, those for slot B and C were synthesized with 4 µg polysomal mRNA from endosperm. The incubations used for slot D and E were synthesized with denatured hybrid structures prepared from 10 µg plasmid pFW 13 as described in Methods. Slot F and G correspondingly from 10 µg plasmid pFW 19. Incubations for slot H and J were supplemented with 10 µg plasmid pBR 322 submitted to the same hybridisation procedure as performed with pFW 13 and pFW 19. The wheat germ incubations used for slots C, E, G and J were immunoprecipiated prior to electrophoresis as described recently (5).

presentative experiment by displaying the gel electrophoretic separation profile of the proteins synthesized in vitro.

It is clearly demonstrated that in the case of plasmid pFW 13 only the mRNA coding for the 19000 M_r zein protein hybridizes with the plasmid DNA while in the case of plasmid pFW 19 only the mRNA coding for the 22 000 M_r protein forms a hybrid with the plasmid. Final proof for the identity of the synthesized proteins is given by a specific antibody precipitation test preceeding the gel electrophoretic separation (5). Control experiments shows that the wheat germ system is completely mRNA dependent and that pBR 322 DNA is not capable of hybridizing with the mRNAs under the conditions of the experiment. The finding that each plasmid contains DNA sequences coding only for one zein RNA is in agreement with the previous recognition of separate mRNAs for the two major zein proteins (5).

The method of analyzing the mRNAs specifically retained in the hybrid of a particular recombinant plasmid by in vitro translation was applied to most of the other zein clones and revealed that clones with DNA inserts coding for the 19 000 M_r zein protein are more frequent.

The pattern of restriction fragments obtained from plasmids pFW 13 and pFW 19 after digestion with restrictions enzymes Eco RI and Pst I are depicted in figure 3. As pBR 322 possesses only one Eco RI site (6) and as the zein specific inserts obviously do not have an Eco RI restriction site, the Eco RI enzyme can be used with advantage for linearizing the recombinant plasmid DNAs. The smaller fragment generated with Pst I very probably represents the complete insert of the recombinant plasmid, as the larger fragment is indistinguishable in size from linearized pBR 322 and as no further fragment could be detected by either polyacrylamide electrophoresis or agarose electrophoresis. Comparison with Hae III marker fragments from λdv I DNA (20) allows an estimation of 580 base pairs and 450 base pairs for the insert of pFW 13 and pFW 19, respectively. For a further characterisation, the DNA of plasmids pFW 13 and pFW 19 or the DNA inserts themselves after their isolation from electrophoresis gels were treated with more restriction enzymes. With plasmid pFW 13 no cleavage sites were found for the enzymes Hind III, Hae III, Hpa II, Hinf and Taq I with the limitation, however, that fragments smaller than 50 base pairs would not have been clearly detected under the conditions of the gel electrophoresis. The only cuts discovered so far are one Bam HI cut and 3 Alu I cuts for pFW 13. The relative size of the various fragments generated as well as the results of the simultaneous digestion with Bam HI and Alu I suggests that the Bam HI cut is located towards one end of the fragment while the 3 Alu I cuts are aligned

Figure 3. Polyacrylamide gel electrophoresis of plasmid DNAs
50 µl incubations containing 100 mM Tris/HCl (pH 7.5) 50 mM NaCl, 5 mM $MgCl_2$ 8 ug plasmid DNA and 5 units restriction enzyme Eco RI (from Boehringer, Germany) or containing 50 mM NaCl, 6 mM Tris/HCl (pH 7.4) 6 mM $MgCl_2$, 6 mM 2 mercaptoethanol, 8 µg plasmid DNA and 5 units restriction enzyme Pst I (from Boehringer) were incubated for 4 hours at 37°C, phenolized and ethanol precipitated. The precipitates were dissolved in 40 µl electrophoresis buffer. 10 µl were applied to 1 mm thick slab gels of 5 % polyacrylamide. Electrophoresis was carried out according to Jeppesen (21). Lanes A and B represent pBR 322 DNA, lanes C and D pFW 13, lane E and F pFW 19 DNA. Lanes A, C and E result from Eco RI assays, lanes B, D and F from Pst I assays. Lane G contains 2 ug of ϕX dvI DNA digested with restriction enzyme Hae III as marker fragments of defined size.

towards the opposite end. Plasmid pFW 19 displays also few cleavages sites. No sites were found for enzymes Bam HI, Hind III, Hae III, Hpa II and Hinf while Alu I generates one cut only. Compared to other DNAs of comparable size the inserts of pFW 13 and pFW 19 contain few restriction enzyme sites, which may reflect the unusual amino acid composition of the hydrophobic zein proteins (22).

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