Research Notes : United States : A method of isolating poly(A)-containing RNA from soybean suspension culture cells for cDNA synthesis and cloning

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1) A method for isolating poly(A)-containing RNA from soybean suspension culture cells for cDNA synthesis and cloning.

Our laboratories are interested in using recombinant DNA techniques to study the genetics of amino acid metabolism in soybeans. An especially useful technique used to study gene regulation is the in vitro synthesis of DNA complementary to mRNA; this cDNA can be used directly as a hybridization probe, or cloned and amplified in a suitable host. The protocol described here was developed for isolating total cellular poly(A)-containing RNA from soybean suspension culture cells of sufficient purity for use as a template for cDNA synthesis. In addition, preliminary experiments were performed to determine the feasibility of constructing a cDNA clone bank in the E. coli cloning vector M13mp7.

Materials and methods

Preparation of plant material. G. max root cell cultures SBe4, GMW, and M24 were maintained as exponentially growing suspension cultures in BS medium (Gamborg et al., 1968). Two-day-old cultures were filtered through a 10-40 µm sintered glass filter and washed with three volumes of ice cold saline. The cell mass was then frozen at -70°C, lyophilized, and stored in airtight containers at 4°C.

RNA extraction. All buffers and glassware were either autoclaved or treated with 0.1% diethylpyrocarbonate.

Total cellular RNA was extracted from lyophilized soybean cells as suggested by Murray and Thompson (1980), with the incorporation of 15mM Na iodoacetate into all solutions. The RNA, pelleted during CsCl/ethidium bromide ultracentrifugation, was dissolved in low-salt buffer plus 15mM Na iodoacetate and extracted several times with saturated 2-propanol to remove ethidium bromide (Maniatis et al., 1982). RNase-free DNase I was added to a concentration of 5 µg/ml, and incubated for 10 minutes at room temperature. The RNA was then ethanol precipitated, pelleted, and redissolved in oligo(dT)-cellulose application buffer.

Poly(A)-containing RNA isolation. Poly(A)-containing RNA was first purified from the total cellular RNA by oligo(dT)-cellulose chromatography as described by Aviv and Leder (1972), using buffers suggested by the supplier of the column matrix (Collaborative Research, Inc.). The poly(A)-containing RNA was further purified by pooling the A260 elution peak, adding phosphate buffer (pH 6.8) to a concentration of 10mM, and applying to a hydroxyapatite column. After extensive washing with 10mM phosphate buffer (pH 6.8) the RNA was eluted with 0.4M phosphate buffer (pH 6.8) (Beachy et al., 1980). The fractions containing the A260 elution peak were again pooled, and added to an equal volume of 2X oligo(dT)-cellulose application buffer, followed by a second round of purification on oligo(dT)-cellulose. The purified poly(A)-containing RNA was then precipitated with ethanol, pelleted, dissolved in distilled and deionized water to a concentration of 1 µg/µl, and stored at -70°C.
**32P-cDNA synthesis.** Single-stranded 32P-cDNA was synthesized as described by Wickens et al. (1978), using 5 µCi of α32P-dCTP, 5 µg of poly(A)-containing RNA, and 100 units of reverse transcriptase. A 2.5 µl aliquot was removed and separated by gel filtration on a 0.5 x 35 cm G50(50-150) sephadex column. Fractions of approximately 0.5 ml were collected and counted by scintillation spectrophotometry.

**Second strand synthesis.** Double-stranded cDNA was synthesized from single-stranded cDNA as described by Wickens et al. (1978), using 5 µCi of 8-3H-dATP, and 50 units of DNA polymerase I. After extraction with chloroform, the entire sample was separated by gel filtration on a 0.5 x 35 cm G50(50-150) sephadex column, collecting 0.5 ml fractions. A 10 µl aliquot of each fraction was removed and counted by scintillation spectrophotometry. The fractions containing the cDNA peak were precipitated with 2-propanol (Maniatis et al., 1982).

**Sl nuclease treatment.** Hairpins and residual single-stranded cDNA were removed in a 500 µl reaction mixture with Sl nuclease as described by Wickens et al. (1978). Resistance to Sl nuclease was determined by gel filtration of 25 µl pre- and post-Sl nuclease aliquots as described for assaying 32P-cDNA synthesis. Sl nuclease treated double-stranded cDNA was then precipitated with ethanol.

**Cloning.** In brief, Sl nuclease treated double-stranded cDNA was end-repaired and blunt-end ligated directly into Hinc-II cleaved M13mp7 RF DNA. The ligation reaction was used to transfect competent E. coli JM103 cultures, which were then plated on media containing IPTG and Xgal. Presumptive clones (colorless plaques) were isolated and confirmed by electrophoresis of native and restriction enzyme digested RF DNAs. A restriction map of one of the clones (wp02) was constructed from restriction analysis.

**Results**

**Poly(A)-containing RNA isolation.** Total cellular and poly(A)-containing RNA preparations were evaluated by staining urea-agarose gels with acridine orange (Locker, 1979). Total RNA preparations contained high molecular weight single-stranded (red-fluorescing) nucleic acid with distinct bands corresponding to approximately 4-6s, 18s, and 30s RNA. No double-stranded (green fluorescing) or RNase-resistant material was visible.

**cDNA synthesis.** The synthesis of cDNA was followed by the incorporation of 32P and 3H counts into the sephadex G50 excluded fraction. The normal yield of single-stranded cDNA was about 100 ng, although yields as high as 750 ng have been obtained. Yields of double-stranded cDNA were usually 200-500 ng.

**Sl nuclease treatment.** The resistance of the double-stranded cDNA to Sl nuclease was determined by the release of 32P and 3H counts from the sephadex G50 excluded fraction into the included fraction. By counting the 32P and 3H channels separately, resistance of 3H-cDNA (double-stranded) as compared with 32P-cDNA (total) was determined; 32P-cDNA was 75% resistant to Sl nuclease, while there was no detectable sensitivity of 3H-cDNA to Sl nuclease.

**Cloning.** A total of 5 confirmed clones were isolated from two separate cDNA syntheses. Restriction analysis showed that the clones were all nearly identical, containing insertions with the same restriction pattern inserted.
in the same orientation. There appear to be slight variations (up to about 10 base pairs) in the length of cDNA inserted; the average is 350 base pairs. The restriction map of wp02 shows that the 350 base pair cDNA insert has 1 Taq I site, 2 Sau 3A I sites, and 3 Hae III sites. There are no Hinc II, Pst I, Sal I, Kpn I, Bam HI, Eco RI, Hind III or Hpa I sites within the cDNA insertion.

Conclusion

A method for isolating highly purified poly(A)-containing RNA from soybean suspension culture cells has been formulated. The poly(A)-containing RNA is free of apparent contamination with DNA, rRNA, and tRNA, and is suitable for use as a template for double-stranded cDNA synthesis. Although cloning this cDNA has met with limited success, presumably because (as was recently shown by J. Messing, personal communication) E. coli JM103 is restriction plus rather than restriction minus, as was previously thought. The recent development of M13 cloning vector hosts that lack restriction activity should eliminate this barrier, making the construction of a soybean cDNA gene bank in M13 a feasible goal.

References


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