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REVERSE TRANSCRIPTION OF 25S SOYBEAN RIBOSOMAL RNA IN THE ABSENCE OF EXOGENOUS PRIMER

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Soybean and other eucaryotic ribosomal RNA's (rRNA's) are shown to serve as templates for the synthesis of cDNA (rcDNA) by avian myeloblastosis virus reverse transcriptase in the absence of exogenously added primer. rRNA present as a contaminant in oligo-(dT)-cellulose bound RNA is efficiently copied into rcDNA that is capable of hybridizing to genomic or cloned ribosomal DNA sequences. The rcDNA can be effectively 'removed' from cDNA preparations by hybridization with excess rRNA. Discrete size classes of rcDNA indicate specific priming and termination sites for rcDNA synthesis. Degradation of template rRNA and the kinetics of rcDNA synthesis suggest that ribonuclease(s) contaminating the reverse transcriptase preparations may "uncover" primers during the course of the reaction.

INTRODUCTION

Synthesis of complementary DNA (cDNA) with the enzyme reverse transcriptase requires a suitable primer as well as the template polynucleotide (1-3). RNA templates are often purified by chromatography on oligo-(dT)-cellulose (4) and the cDNA reaction is subsequently primed by the addition of oligo-(dT). It is generally assumed that contaminating rRNA, which may be present even after several cycles of oligo-(dT) chromatography, is not copied by reverse transcriptase under standard conditions. rRNA has been copied by reverse transcriptase after the addition of poly A "tails" (5), by random priming with fragments of calf thymus DNA (6), or specific priming with fragments of cloned rDNA (7).

We present data which show that a number of eucaryotic rRNA's (soybean, pea, wheat, rabbit) can be copied by reverse transcriptase in the absence of exogenously added primers. rRNA present as a contaminant in oligo-(dT)-cellulose bound RNA is copied under standard reaction conditions. Our data suggest that the mechanism of synthesis of rcDNA may involve partial degradation of the rRNA by ribonucleases (RNases) associated with the reverse transcriptase preparations generating specific priming sites within the molecules.

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MATERIALS AND METHODS

RNA AND DNA Isolation. Soybean (Glycine max var. Prize) RNA was isolated from total cytoplasmic polysomes of 21 day-old root nodules as previously described (8). Polysomal RNA was fractionated into (dT)-bound and -unbound fractions by passage over oligo-(dT)-cellulose (Collaborative Reserch) (4,8). Ribosomal RNA's were isolated from the oligo-(dT)-unbound material after sedimentaion through 10-30% sucrose gradients (100mM NaCl, 10mM Tris-HCl, pH 7.4, 1mM Na_2EDTA, 0.1% SDS[1]) in a Spinco SW41 rotor, 30,000 rpm for 12 hours at 20°C. The UV tracing of a typical gradient profile indicated a mass ratio of 1.05 : 2.00 for 17S : 25S (data not shown). Total RNA from wheat embryos and rabbit reticulocytes was prepared as above. RNA from pea seedlings and E. coli was obtained using a cesium chloride procedure (9). DNA was extracted from soybean embryos as described previously in detail (10).

Synthesis of cDNA. cDNA was synthesized to oligo-(dT)-bound and -unbound RNA under a variety of reaction conditions, all of which generated rcDNA. "Standard reaction conditions" (as referred to in the text) were 50 mM tris-HCl (pH 8.3 at 37°C), 70 mM KCl, 9 mM $MgCl_2$, 20 mM β-mercaptoethanol and 400 μM of each deoxyribonucleotide triphosphate, including the radiolabelled dCTP. Reaction volumes were 25 to 100 μl, RNA concentrations were 5 to 150 μg per ml and oligo-$(dT)_{12-18}$ (Miles) was at 20 to 50 μg per ml when present. [^{3}H]dCTP (20Ci/mM) and [^{32}P]dCTP (various specific activities) were from New England Nuclear. Reactions were initiated by the addition of the reverse transcriptase at 1 to 5 units per μg of RNA. Incubations were at 37°C for various times. Reactions were terminated by the addition of Na_2EDTA (pH 8.0) to 20 mM. RNA was hydrolyzed by NaOH (0.3 M at 60°C for 60 min. or at 37°C for 16 hr.), the mixture neutralized, extracted with chloroform: isoamyl alcohol (24:1, v/v) and passed over a Sephadex G-100 (Pharmacia) column (0.8 x 30 cm.) in 100 mM Na-acetate, 10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA. The excluded volume fractions were pooled and ethanol precipitated. The avian myeloblastosis virus reverse transcriptase used in this study (lot numbers G1178 and G1378) was obtained from Dr. J. Beard (Life Sciences, Inc., St. Petersburg, Florida 33707, U.S.A.).

Gel Electrophoresis and Hybridization. Eco RI (Boehringer Mannheim) digestions were done using 4 units of enzyme per μg of DNA for 60 minutes. Agarose (1 to 1.5%) gel electrophoresis (vertical slab) was carried out in 40 mM Tris-HCl (pH 7.5), 5 mM Na-acetate, 1 mM Na_2EDTA at 30 volts (constant voltage) for 12 to 18 hours with recirculating buffer. DNA was blotted on to nitrocellulose paper (Millipore, HAWP) by the method of Southern (11). Hybridizations were done at 70°C for 12 to 48 hours in 0.45 M NaCl, 0.045 M Na_3-citrate, 0.2% BSA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 100 g/ml poly A, 200 μg/ml sonicated, heat denatured calf thymus DNA and 0.1% SDS. Methyl mercury hydroxide gel eletrophoresis was done as described by Bailey and Davidson (12) using 1.5% agarose and 7.5 mM methyl mercury hydroxide (Ventron Corp., N.Y.) with appropriate safety precautions. Autoradiography was performed using pre-fogged Kodak X-omat film at -70°C (13). Agarose gels were exposed to film at 5°C. rRNA was 5' end labeled with [^{32}P]ATP (3000-6000 Ci/m mol, New England Nuclear) using polynucleotide kinase (New England Biolabs) after partial alkaline hydrolysis (14). The genomic clone of soybean rDNA (Ch4A Gm9) has been characterized in detail (10).

[1]Abbreviations used: BSA, bovine serum albumin; Na_2 EDTA, disodium salt of ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

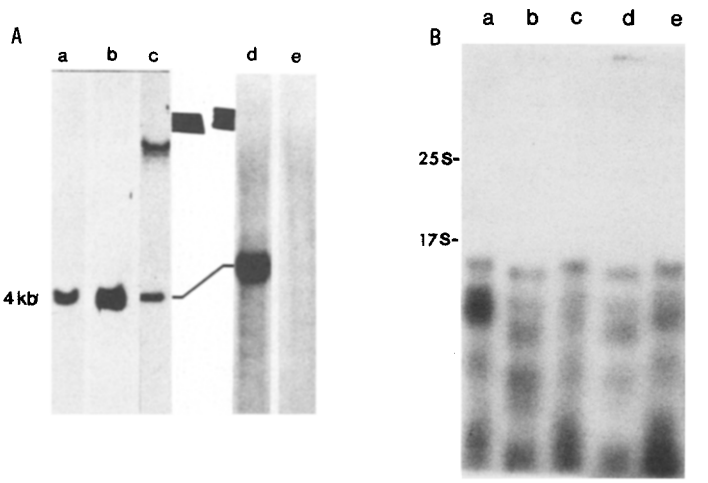

Figure 1,A. Hybridization of rcDNA and rRNA to genomic DNA sequences. Soybean DNA (tracks a,b,d, and e; 10 µg each) and soybean rDNA clone CH4A Gm9 (track c, 0.5 µg) were digested with Eco RI, electrophoresed in agarose gels and transferred to nitrocellulose paper. The hybridization probes were (tracks a,c,d, and e) ^{32}P cDNA made to oligo-(dT)-cellulose bound soybean root nodule RNA (specific activity approx. 5 x 10^6 cpm/µg) and (track b) ^{32}P end labeled rRNA (sp. act. approx. 10^6 cpm/µg). Tracks d and e are identical to track a but with a shorter gel run and a longer film exposure. Track e is hybridized with cDNA which had been pre-hybridized with a 550 fold (by mass) excess of unlabeled rRNA.

Figure 1,B. Size analysis of soybean RNA reverse transcription products. cDNA reactions were assembled without added primers using root nodule oligo-(dT)-cellulose bound RNA (track a), 17S rRNA (tracks b and c), and 25S rRNA (tracks d and e). 10,000 cpm of reaction products were loaded on to each track with (b and d) or without (a, c and e) prior base treatment (60°C, 30 minutes, 0.3M NaOH). Positions of 17S and 25S rRNA were visualized by ethidium bromide fluorescence of rRNA added to each sample.

RESULTS AND DISCUSSION

We observed that the cDNA prepared to oligo-(dT)-bound RNA from soybean root nodules intensely hybridized to two closely associated bands of Eco RI cut genomic DNA (fig. 1A, lane a). Based on the specific activity of the probe, the amount of DNA loaded on the gel and the intensity of hybridization, the hybridized sequences appeared to be highly reiterated. Purified end-labeled rRNA hybridized to the same major bands (fig. 1A, lane b) suggesting that the cDNA preparation contained sequences complementary to rRNA. This was confirmed (fig. 1A, lane c) by hybridization of total cDNA to the Eco RI cut genomic clone Ch4A Gm9 of soybean rDNA (10).

Reverse transcriptase copies both soybean rRNA's in the presence or absence of oligo(dT) or Actinomycin D, with or without heat treatment, with a mass conversation of up to 25% of template into rcDNA. Reverse transcription of rRNA's is not specific to soybean root nodule rRNA but was also observed

with soybean leaf and hypocotyl rRNA (data not shown) as well as with the rRNA's of several other eucaryotes, pea, wheat and rabbit (see below). We have recently isolated a recombinant clone of soybean double stranded rcDNA (our unpublished data) from the library of cDNA clones.

These data show that oligo-(dT) primed cDNA reaction products cannot be assumed to be free of sequences complementary to rRNA. In many applications, e.g. screening 'libraries' of recombinant molecules, the presence of rcDNA could lead to the misinterpretation of hybridization data. It is therefore desirable to find reverse transcriptase reaction conditions under which contaminating rRNA's are not copied or to eliminate the rcDNA's from the reaction products. Repeated passage of total cDNA over poly(A)-Sepharose (15) resulted in the elimination of most but not all of the rcDNA from the bound fraction (data not shown). We have found that prehybridization of the cDNA with a 500 fold (or greater) excess of rRNA eliminates any contribution of the rcDNA to subsequent hybridizations (fig. 1A, lanes d and e). This is accomplished by adding unlabeled rRNA to the cDNA and incubating for 30-60 minutes under the same conditions as the subsequent filter hybridization. Williamson and Lloyd (16) have observed rcDNA in their preparations of Dictyostelium cDNA and have used pre-hybridization with rRNA to eliminate the rcDNA from subsequent hybridization. It must be noted that "contamination" of the rRNA preparations with messenger RNA sequences could result in the prehybridization of desirable sequences as well as the rcDNA. Using recombinant rDNA as the competitor circumvents this problem. Hybridization of total cDNA preparations to cellular RNA preparations separated on methyl mercury hydroxide gels and blotted onto diazotized paper (17) can be used as a sensitive assay for the presence of contaminating rcDNA.

The mechanism responsible for reverse transcriptase copying of rRNA was investigated by analysis of first, the sizes of the rcDNA's, second, the time course of the rcDNA reactions and third, the effect of reverse transcriptase on the rRNA template. The products synthesized using 17S, 25S or oligo-(dT)-cellulose bound RNA (without added primer) when analyzed on methyl mercury hydroxide gels are essentially of the same size classes, ranging from approximately 200 to 2000 bases (fig. 1B). It is surprising that no counts are associated with molecules of 17S size or larger and that base treatment shortens the labeled molecules by only about 100 bases. These observations suggest that synthesis does not occur on intact 25S molecules or that the template (and/or primer) is being degraded during the reaction.

The 25S soybean rRNA reaction shows radically different kinetics than the reaction of oligo-(dT)-cellulose bound RNA primed with oligo-(dT) (fig. 2A). cDNA was also synthesized in reactions without added primer using poly

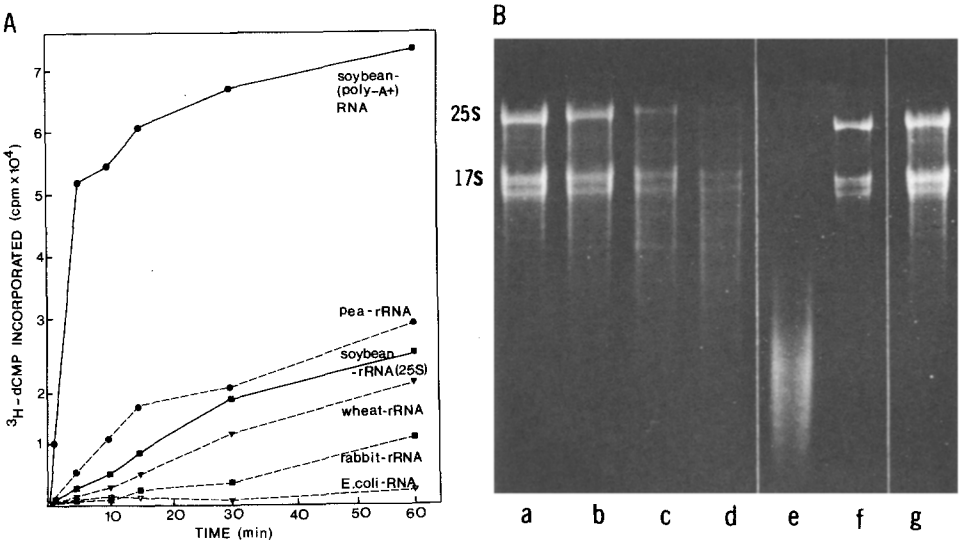

Figure 2,A. Kinetics of rcDNA and cDNA synthesis. Standard cDNA reactions were assembled in 30 µl containing 4 µg of RNA and [^{3}H]dCTP. 0.6 µg of oligo-$(dT)_{12-18}$ was added to the poly A^+ RNA reaction. At the indicated time points 3 µl aliquots were removed and processed for TCA-precipitable incorporation as described by Friedman and Rosbach (25).

Figure 2,B. Degradation of the rRNA template during reverse transcription. cDNA reactions were assembled with 5 µg 25S rRNA per 50 µl reaction. Tracks a through e: 5U reverse transcriptase per reaction incubated at 37°C for 1 min (a), 5 min (b), 10 min (c), 20 min (d) and 55 min (e). Track g as in e but without reverse transcriptase. Track f, 3 µg untreated rRNA. Reactions were terminated with EDTA and precipitated with ethanol. The precipitates were dissolved in 25 mM methyl mercury hydroxide prior to loading on to the methyl mercury hydroxide gel.

A(-) RNA from pea, wheat and, to a lesser extent, from rabbit reticulocytes. Pre-treatment of the RNA's with DNAase (data not shown) had no effect on the kinetics indicating the small fragments of DNA are not responsible for the priming events. The incorporation of a few counts into TCA precipitable material using E. coli rRNA as templates under identical conditions to that of eucaryotic rRNA's (fig 2A) implicates structural features of the latter molecules as being involved in priming. Figure 2A also shows that short reaction times (e.g., 10 to 15 minutes) may be useful to minimize contamination of cDNA preparations with rcDNA.

Figure 2B shows that the rRNA template is progressively degraded by components of the reverse transcriptase enzyme preparation. After 55 min of reaction time, there are no detectable full sized template molecules (fig. 2B, lane e). This extensive degradation is dependent upon the addition of reverse transcriptase (lane g). The addition of components reported to reduce ribonuclease H activity (18) reduced the degradation by only a small

amount (data not shown). This extensive degradation explains the lack of radioactive molecules larger than 17S and the small effect of base treatment on the mobility of the cDNA reaction products (fig. 1B).

It is known that many rRNA molecules contain internal nicks (19) which only become evident when the molecules are analyzed under denaturing conditions (e.g., methyl mercury hydroxide gels, see fig. 2B). Such nicks could be responsible for priming the rcDNA. The relatively slow kinetics (fig. 2A) could be due to the difficulty the enzyme has working through the kinetic barriers of secondary structure (3) and/or methylated bases (7,20,21), both features known to occur in rRNA's (22). Priming may also be done by 5.8S RNA molecules, known to be hydrogen bonded to the larger rRNA's of HeLa cells (23) and yeast (24). Small RNA's are not visible in figure 2B but 5.8S and other species of small RNA's are readily detectible when 5 to 10 μg of soybean rRNA is analyzed under the same conditions (data not shown). If the primers and templates are separate molecules held together by hydrogen bonding of complimentary sequences, then denaturation prior to reverse transcription should significantly reduce the level of rcDNA synthesis. Heat treatment of soybean 25S rRNA prior to the reverse transcriptase reaction had little effect on the synthesis of rcDNA.

An alternative source of primers would be the action of the RNase(s) contaminating the reverse transcriptase preparations. RNase, acting as an exonuclease, could "uncover" a primer if it stopped at an area of secondary structure. At this point, reverse transcriptase could rapidly copy the template strand of RNA until it too encountered a kinetic barrier (or reached the end of the molecule). The RNase activity associated with reverse transcriptase could clip the RNA template at a secondary structure barrier and generate another primer. This model predicts that the progressive degradation of the rRNA (fig. 2B, lanes a through e) is required for rcDNA synthesis.

The distinct size classes of rcDNA molecules (fig. 1B) may reflect the distances between the priming sites and the major kinetic barriers for reverse transcriptase. This would imply a higher degree of structural similarity between the soybean 17S and 25S rRNA molecules since the rcDNA's produced from each rRNA were of similar size classes (fig. 1B). The reproducible 2:1 mass ratio of 25S to 17S in the neutral sucrose gradients from which the rRNA's were isolated argues against significant contamination of the 17S with fragments of 25S molecules. Structural similarities between the 17S and the 25S rRNA's are also indicated by the spacings of certain restriction endonuclease sites within the cloned rRNA genes (10).

It is possible that the selective utilization of RNases with reverse transcriptase could be employed to investigate structural features of RNA molecules.

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