SYNTHESIS OF A COMPLEMENTARY DNA TO RAT LIVER ALBUMIN mRNA

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SUMMARY. A complementary DNA (cDNA) to purified rat liver albumin mRNA has been synthesized by means of a viral reverse transcriptase. The most important synthetic reaction parameters affecting cDNA product size were a nucleotide substrate concentration of at least 50 μM and a mRNA template concentration of about 25 μg per ml. At least 50 μg per ml of actinomycin D was required to prevent significant double-stranded cDNA synthesis. The final albumin cDNA produced was estimated to be essentially a full-length copy of its template by sedimentation in alkaline sucrose gradients.

The preparation of highly purified rat liver albumin mRNA (1) has permitted the synthesis of a radioactively labeled complementary DNA (cDNA) with a viral reverse transcriptase. Rat albumin cDNA could be employed as a sensitive hybridization probe to quantitate mRNA levels in various physiological conditions. The specific cDNA could also be employed for quantitation of structural genes, examination of mRNA processing, and determination of nucleotide sequences. In many studies, it is desirable to use long or full-length cDNA preparations. Synthetic reaction conditions for long cDNAs against several mRNA species (2-4), however, appear to vary with respect to certain reaction parameters. These findings suggest that the nucleotide sequence and secondary structure of a particular mRNA species may require a unique set of reaction conditions for the production of a full-length cDNA.

In this communication, the reaction conditions required for the synthesis of long and possibly full-length copies of albumin mRNA as well as total liver poly(A)-containing mRNAs are reported. The native rat albumin protein contains 575 amino acids (5), and would be translated from a molecule of mRNA containing at least 1,800 nucleotides.

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MATERIALS AND METHODS

Preparation of mRNA. Rat liver albumin mRNA was purified by specific polysome immunoprecipitation and poly(U)-Sepharose affinity chromatography as described previously (1). Total liver poly(A)-containing mRNAs were prepared by phenol-chloroform extraction and poly(U)-Sepharose affinity chromatography (6).

Reverse Transcriptase. The RNA-dependent DNA polymerase from avian myeloblastosis virus (AMV) was generously supplied by Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, FL) through the auspices of National Institutes of Health. The enzyme was supplied in a solution of 200 mM potassium phosphate at pH 7.2, 2 mM dithiothreitol, 0.2% Triton X-100, and 50% glycerol.

Synthesis of cDNA. Sufficient [^{3}H]dCTP and [^{3}H]dGTP (27 and 37 Ci/mmol, respectively, from New England Nuclear) to yield the desired final reaction concentration were evaporated to dryness at 0° under nitrogen in a 0.3 ml conical reaction vial. The final reaction volume of 50 µl was supplemented with dATP and dTTP. The concentrations of the nucleotide substrates were varied from 20 µM to 1500 µM; with dCTP, dGTP, and dATP always at the same concentration, and dTTP never less than 200 µM. The final reaction mixture also contained 50 mM Tris-HCl (pH 8.3), 20 mM dithiothreitol, 6 mM $MgCl_2$, 100 mM NaCl, 30 µg per ml of oligo(dT)$_{12-18}$ (Collaborative Research), and 100 units per ml of AMV reverse transcriptase. The mRNA concentration was varied from 6 µg per ml to 120 µg per ml. Actinomycin D was supplemented from 20 µg per ml to 200 µg per ml. The final reaction mixture was incubated at 37° for varying times up to 2 hrs. For the purpose of examining individual reaction parameters, a standard reaction mixture was employed, which was adjusted to 50 µM in dCTP, dGTP, and dATP, 25 µg per ml in mRNA, 50 µg per ml in actinomycin D, and incubation was at 37° for 20 min.

Following cDNA synthesis, the final reaction mixture was then adjusted to 1% in sodium dodecyl sulfate and 10 mM EDTA at pH 7.0. This solution was added to a Sephadex G-50 column (0.7 x 40 cm) which had been equilibrated in 100 mM NaCl, 10 mM EDTA, and 20 mM HEPES at pH 7.5. Elution was with this same buffer, and the cDNA in the excluded fraction was collected. The cDNA fraction was adjusted to 0.2 M NaOH, incubated at 70° for 15 min, then cooled quickly to 0°. After neutralizing the solution with 1 M HEPES at pH 7.0, this cDNA fraction was adjusted to 0.2 mg per ml with yeast tRNA and 0.2 M NaCl, and precipitated with 2.5 volumes of ethanol at -20° overnight.

In many experiments, cDNA reaction products were monitored by measuring trichloroacetic acid-precipitable radioactivity in the presence of 100 µg of calf thymus DNA (Sigma) or bovine serum albumin (Sigma) added as a carrier. Assuming an equimolar composition in each base, the specific radioactivity of various cDNA preparations was estimated to be 4 x 10^4 cpm per ng.

Centrifugation of cDNA in Alkaline Sucrose Gradients. An aliquot containing about 2 x 10^5 cpm of cDNA (which had been eluted from Sephadex G-50 and heated in 200 mM NaOH, as described above) was layered on a 5-20% (w/v) linear sucrose gradient containing 200 mM NaOH, 2 mM EDTA, and 100 mM NaCl. The gradients were centrifuged at 280,000 xg_{max} for 20 hrs at 4° in a Beckman SW41 rotor. Fractions of 0.4 ml were collected, neutralized, and the radioactivity was measured with TT-21 scintillation fluid (Yorktown Research). The gradients were characterized by the sedimentation of a preparation of sheared rat DNA having an average length of 450 nucleotides. DNA was prepared, sheared through a pressure cell, and the average fragment length estimated by sedimentation in isokinetic alkaline sucrose gradients as described by others (7-9).

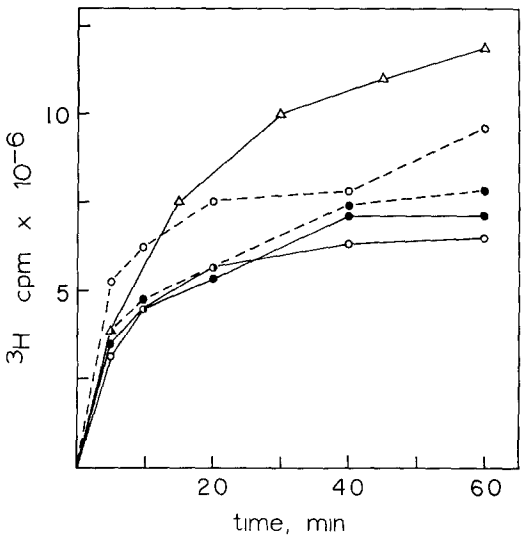

Figure 1: The Effect of Actinomycin D Concentration on the Time Course of cDNA Synthesis.

Total liver mRNA was incubated for 60 min at 37° in the standard cDNA synthesis reaction mixture with 20 µg/ml (Δ——Δ), 50 µg/ml (●---●), 100 µg/ml (●——●), or 200 µg/ml (O——O) of actinomycin D. An incubation at 46° with 50 µg/ml (O---O) of actinomycin D was also performed. Aliquots of 10 µl were removed at the indicated times and the trichloroacetic acid-precipitable radioactivity was measured.

Hybridization Assay. Hybridization reactions of excess mRNA to [^{3}H]cDNA were performed at 68° essentially as described (10), except that the reaction mixtures contained 0.5 M NaCl and 9 µg of yeast tRNA (PL Laboratories).

Thermal Denaturation of cDNA:mRNA Hybrids. Hybridization reactions were performed with sufficient RNA to hybridize more than 85% of a 5,000 cpm sample of [^{3}H]cDNA. Hybrids were bound to 1 ml of hydroxyapatite (BioRad) in a water-jacketed column at 60° and thermal elution was performed essentially as described (11). The cDNA eluted at each temperature was precipitated with trichloroacetic acid in the presence of added calf thymus DNA, and radioactivity was measured (11).

RESULTS AND DISCUSSION

A preliminary investigation of the reaction requirements showed that the production of acid precipitable radioactive cDNA was dependent on the presence of DNA primer, mRNA template, and the presence of all four deoxynucleotide substrates. The absence of actinomycin D resulted in the synthesis of more cDNA product, as reported by others (11,12); and the additional product is apparently due to double-stranded DNA synthesis (12-14).

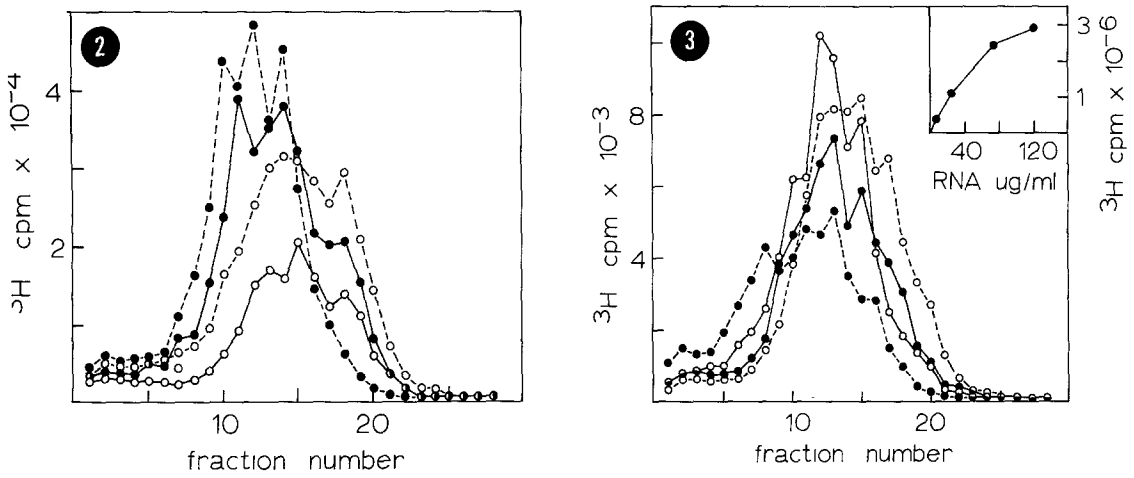

Figure 2: Effect of Deoxynucleotide Substrate Concentration on cDNA Synthesis.

Total liver mRNA was incubated in the standard cDNA synthesis reaction mixture at different deoxynucleotide substrate concentrations. In each reaction, the four substrates were at equimolar concentrations of 20 μM, (●---●); 50 μM, (●——●); 500 μM, (O---O); and 1500 μM, (O——O). After the reaction was completed, the cDNA product was eluted from Sephadex G-50, heated in alkali, and examined by sedimentation on parallel alkaline linear sucrose gradients. The direction of sedimentation is from left to right.

Figure 3: Effect of mRNA Template Concentration on cDNA Synthesis.

Different concentrations of total liver mRNA were incubated under the standard reaction conditions, and the cDNA product was analyzed by alkaline sucrose sedimentation on parallel alkaline linear sucrose gradients. The direction of sedimentation is from left to right. The concentrations of mRNA template examined were 6 μg/ml (●---●); 25 μg/ml, (O---O); 75 μg/ml, (●——●); and 120 μg/ml, (O——O). The inset shows the yield of cDNA at the different template concentrations.

The time course of cDNA synthesis at different actinomycin D concentrations was investigated (Fig. 1). At concentrations of 50 μg per ml or greater, no further significant increase in cDNA production was observed after 40 min. Furthermore, only 2-8% of the material in these cDNA preparations was resistant to digestion with the single-strand specific S1 nuclease, indicating that a significant amount of double-stranded product was not produced at these actinomycin D levels. Examination of these cDNAs on alkaline sucrose gradients revealed no apparent differences in size distribution (data not shown). However, the cDNA produced at 20 μg per ml of actinomycin D contained variable,

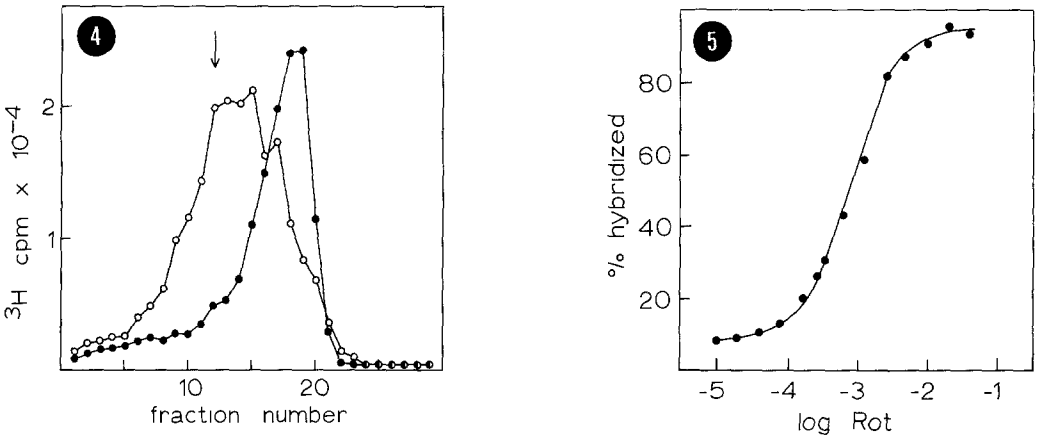

Figure 4: Size Distribution of Albumin cDNA on Alkaline Sucrose Gradients.

Preparations of albumin cDNA (●——●) and cDNA to total liver mRNA (○——○), which had been synthesized under reaction conditions optimized for long length were examined on separate parallel alkaline linear sucrose gradients. The direction of sedimentation is from left to right. The arrow indicates the position of the 450 nucleotide sheared rat DNA marker.

Figure 5: Hybridization of Albumin cDNA with Purified Albumin mRNA.

RNA excess hybridizations of highly purified albumin mRNA to albumin cDNA and detection of hybrids by S1 nuclease resistance were performed. Each reaction mixture contained 8.6 ng or 1.1 ng of mRNA with 2000 cpm of cDNA; and incubations of 30 sec to 4.3 hr were performed.

higher levels of S1 nuclease-resistant (double-stranded) material. Increasing the incubation temperature to 46° (with 50 µg per ml of actinomycin D) stimulated the synthetic reaction rate, as reported by Monahan *et al.* (2). However, the size distribution of the cDNA product was not altered.

Recent reports have demonstrated a significant effect of the concentrations of deoxynucleotide substrates on the length of the cDNA product (2-4). The size distribution of cDNA synthesized at different substrate concentrations with a total liver mRNA template is shown in Fig. 2. The gradient pattern did not change significantly at deoxynucleotide concentrations from 50 to 1500 µM. However, an increased amount of relatively smaller material is produced at lower substrate concentrations.

The effect of mRNA template concentration on the size distribution of the cDNA product was investigated (Fig. 3). The cDNA of greatest average

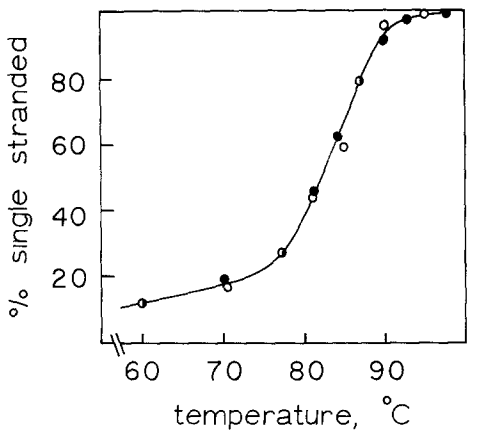

Figure 6: Thermal Denaturation of mRNA:cDNA Hybrids.

The thermal stability of albumin mRNA:cDNA hybrids (●——●) and total liver mRNA:cDNA hybrids (O——O) was assessed by melting off hydroxyapatite as described in "Materials and Methods".

length was produced at mRNA levels of 20-30 µg per ml. Lower concentrations yielded less product, and it showed a reduced average length. At higher concentrations of template, the average size distribution of the cDNA was also smaller, even though the acid-precipitable product yield was much greater.

Sedimentation profiles of the cDNA preparations on alkaline sucrose gradients (calibrated with sheared rat DNA standards) are shown in Fig. 4. Most of the albumin cDNA material sedimented in a size range consistent with molecules containing 1000-2000 nucleotides. By comparison, the cDNA to total liver mRNA was more heterogeneous in length, ranging from about 200-2,000 nucleotides. Most of the cDNA sedimented faster than a 450 nucleotide sheared rat DNA standard.

The hybridization kinetics of the albumin cDNA with purified albumin mRNA are shown in Fig. 5. Nearly complete hybridization occurred within a 100-fold range of Rot values and attained a final level of 95% or more of the input cDNA. Hybridization to 10-fold higher Rot values did not increase the quantity of hybrid formed. A Rot1/2 of 7.94×10^{-4} mol sec l^{-1} can be calculated for the hybridization of albumin mRNA with its cDNA.

The thermal stability profiles of albumin mRNA:cDNA hybrids and total liver mRNA:cDNA hybrids are shown in Fig. 6. Melting of the hybrids off hydroxyapatite showed a Tm of about 86° in both cases, with relatively sharp thermal transitions, indicating that faithful transcription had occurred.

A long single-stranded cDNA copy of rat liver albumin mRNA has been prepared by individually optimizing various parameters of the synthetic reaction mixture. A nucleotide substrate concentration of about 50 μM and an mRNA template concentration of about 25 μg per ml were required. Short incubation times and actinomycin D at a level of 50 μg per ml or greater were required to suppress double-stranded cDNA production. Under these conditions, a yield of 170 ng of cDNA per μg of mRNA was obtained. The albumin cDNA is a faithful copy of its template, and they hybridize with reaction kinetics which are consistent with a homogeneous mRNA preparation.

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