SYNTHESIS OF COMPLEMENTARY DNA FROM LENS mRNA WITH RNA-DEPENDENT DNA POLYMERASE*

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Summary: Lens 10S and 14S mRNAs, isolated by zonal centrifugation, were shown to function as templates for the synthesis of complementary DNA (cDNA) with RNA-dependent DNA polymerase of avian myeloblastosis virus (AMV). The cDNA products, synthesized with the lens 10S and 14S mRNA templates, gave sedimentation constants of 7.6S and 8.3S, respectively. The complementarity of the cDNAs to their specific RNA templates was demonstrated by hybridization experiments.

Introduction: The discovery of RNA-dependent DNA polymerase associated with oncogenic viruses (1,2) and the subsequent demonstration of its presence in mammalian tissue (3,4) have implications for the study of tumors in higher animal systems (5,6) and gene amplification (7) as well as normal development and differentiation (8). The feasibility of using AMV RNA-dependent DNA polymerase to synthesize complementary DNA has been demonstrated with globin (9-11) and immunoglobin (12) mRNAs.

The avascular lens provides another interesting mammalian system for such studies. The 10S and 14S mRNAs from lens have been isolated by zonal centrifugation by Bloemendal and coworkers (13) and have been shown to be capable of producing the B and A polypeptide subunit chains of alpha crystallin, respectively (14,15).

This communication reports experiments which demonstrate that lens mRNAs isolated by zonal centrifugation can serve as templates for the synthesis of complementary DNA in the presence of oligo(dT) primer with AMV RNA-dependent DNA polymerase. The cDNAs synthesized exhibit specific complementarity with their corresponding lens mRNA templates on the basis of hybridization experiments.

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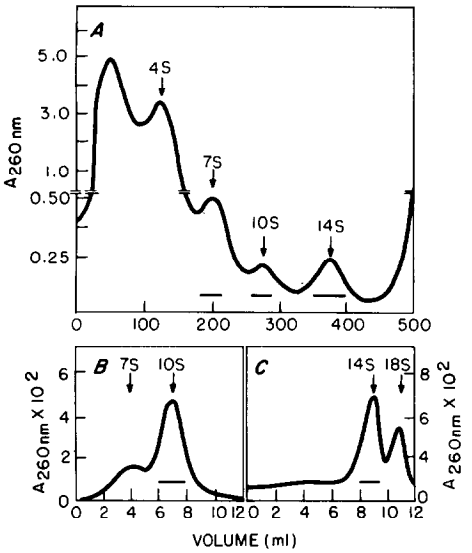


Figure 1. Zonal centrifugation of polysomal RNA from lens polyribosomes and recentrifugation of the 10S and 14S RNA fractions. Lens polyribosomes were prepared from 300 calf lenses as described previously (23). The polysomal RNAs were treated with 1% SDS at 37°C for 8 minutes (13) and then resolved in a 0 to 30% equivolumetric sucrose gradient (16) containing 0.05 M Tris-HCl pH 7.4. Centrifugations were performed in an International B-XXX zonal rotor at 50,000 rpm for 15.5 hrs. at 4°C. The separated polysomal 10S and 14S RNA fractions were recentrifuged through 5 to 19% isokinetic sucrose gradients (24) containing 0.05 M Tris-HCl pH 7.4 in a SW 41 Spinco rotor for 13 hrs. at 41,000 rpm at 4°C. Bars delineate the volumes which were pooled. (A) Zonal centrifugation profile, (B) and (C) recentrifugation profiles of the 10S and 14S polysomal RNAs, respectively.

Results: Lens polysomes, isolated from calf lens homogenates, were fractionated by zonal centrifugation through equivolumetric sucrose gradients after treatment with 1% SDS (16). As shown in Fig. 1A, the polysomal RNAs were resolved into several different size species as previously reported by Berns et al. (13). The 10S and 14S RNA species were then recentrifuged through isokinetic sucrose gradients. Such experiments revealed a very considerable purification of each of these RNA fractions (Fig. 1B and 1C). The 10S and 14S peaks were individually pooled, concentrated, and used for subsequent translation and transcription assays. Comparable to the results obtained by other investigators (17), both 10S and 14S RNA fractions were found to stimulate ^{3}H-leucine incorporation into CCl_3COOH precipitable polypeptide material

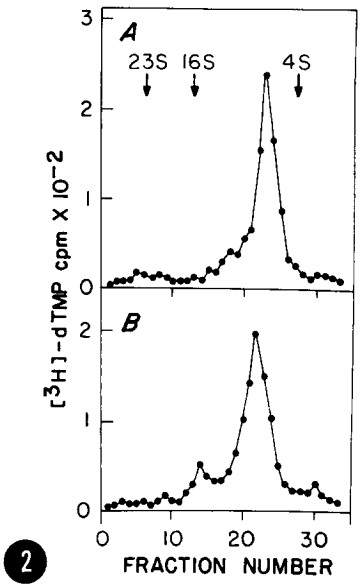
Table I. RNA-dependent DNA polymerase activity with various mRNAs.

mRNA Template Size	Conc. (μg)	Reaction Condition	^{3}H-dTMP Incorporation (p moles)
Lens 10S mRNA	2.0	Complete	12.21
	2.0	- oligo(dT)	0.56
	2.0	+ Actinomycin D	7.41
	2.0	+ RNase A	0.61
	0	Complete	0.46
Lens 14S mRNA	2.0	Complete	4.38
	2.0	- oligo(dT)	0.52
	2.0	+ Actinomycin D	2.36
	2.0	+ RNase A	0.45
Lens 7S RNA	2.0	Complete	0.36
	2.0	- oligo(dT)	0.37
Globin 9S mRNA	2.0	Complete	5.94
	2.0	- oligo(dT)	0.34

AMV RNA-dependent DNA polymerase reactions were performed as previously described (9). The basic assay mixture of 100 μl contained the following in μ moles: Tris-HCl (pH 8.3), 5.0; $MgCl_2$, 0.6; dATP, dCTP and dGTP, 0.02; ^{3}H-dTTP (specific activity, 1800 cpm/p Mole), 0.004; KCl, 5.0; an appropriate enzyme dilution and the indicated template RNA concentrations. The following additional components were used in certain assays as indicated in the Table: oligo(dT), 0.4 μg; Actinomycin D, 100 μg/ml; RNase A, 10 μg.

with a Krebs-II-ascites cell-free system. The 14S RNA has been shown to be a messenger for the synthesis of alpha crystallin A chains and the 10S lens mRNA for the B chains by both in vitro (14) and in vivo (15) experiments.

As shown in Table 1 when the AMV RNA-dependent DNA polymerase was tested with 10S and 14S lens mRNA, approximately a 22- and 9-fold stimulation of ^{3}H-dTMP incorporation into CCl_3COOH precipitable material was observed, respectively. The reaction was essentially dependent upon oligo(dT). No incorporation of ^{3}H-dTMP was found with the lens 7S RNA fraction. Globin 9S mRNA, under the same conditions, was found to give a 17-fold stimulation of ^{3}H-dTMP incorporation. Preincubation with



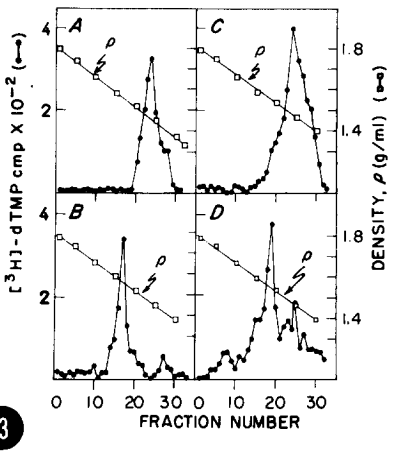


Figure 2. Sucrose gradient centrifugation profiles of DNA products. DNA products were obtained with AMV RNA-dependent polymerase with either the 10S or 14S mRNAs as described in Table I. After incubation for 50 minutes at 37°C, the reaction mixture was extracted with phenol-cresol-chloroform, and the organic phase back extracted. The combined aqueous phase was passed through a Sephadex G-50 column (1.1 x 80 cm). The DNA product was adjusted to 0.4 M NaCl, precipitated with ethanol, dissolved in 0.003 M EDTA and incubated with 0.3 M KOH at 37°C for 18 hrs. After neutralization with HCl it was stored at -84°C. Sedimentation analysis of the DNAs were performed in 15 to 30% linear sucrose gradients containing 0.01 M Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM EDTA and run at 38,000 rpm for 20 hrs. at 4°C in a SW 41 Spinco rotor. Arrows indicate the sedimentation peak position of standard RNA markers in a parallel gradient. (A) DNA synthesized from 10S lens mRNA template and (B) DNA synthesized from 14S lens mRNA template.

Figure 3. Cs_2SO_4 bouyant density equilibrium centrifugation profiles of cDNAs and their hybrids with lens 10S and 14S mRNA templates. Hybridization experiments were performed in 50 µl using 0.4 µg of mRNA and 8×10^{-5} µg of DNA in the presence of 50% formamide and heated at 82°C for 10 minutes. After quick cooling, 0.1 volume of 4 M NaCl was added and the reaction mixture was allowed to stand for 24 hrs. at 37°C. After incubation the reaction mixture was made up to 0.0015 M EDTA and 50% saturation with respect to Cs_2SO_4. The centrifugations were then carried out at 20°C in either a Spinco 65 rotor at 38,000 rpm for 70 hrs. or in a Spinco SW 41 rotor at 32,000 rpm for 65 hrs. (A) 7.6S cDNA and (B) its hybrid with 10S lens mRNA, (C) 8.3S cDNA and (D) its hybrid with 14S mRNA.

RNase A alone eliminated more than 90% of the template activities for both 10S and 14S mRNA fractions. With either mRNA, the addition of actinomycin D caused approximately a 50% suppression of ^{3}H-dTMP incorporation into CCl_3COOH precipitable

material. In all experiments, no substantial incorporation of ^{3}H-dTMP was observed if the other deoxyribonucleotides substrates were omitted.

An estimation of the size of the synthesized cDNAs obtained with the lens mRNA fractions was made on linear sucrose gradients calibrated with standard RNA markers. Such experiments gave sedimentation constants for the cDNAs of 7.6S and 8.3S for the 10S and 14S mRNA templates, respectively (Fig. 2A and B).

The specific complementarity of the cDNAs was examined by hybridization with the mRNAs which had served as their respective templates. Fig. 3A and 3C shows that the cDNA products banded in a Cs_2SO_4 bouyant density gradient at a density of approximately 1.46 g/ml, a density region characteristic of DNA. However, a definitive displacement of the cDNA to a heavier density of approximately 1.56 g/ml, indicative of RNA-DNA hybrids, resulted after hybridization of the 7.6S cDNA with its 10S mRNA template (Fig. 3B). After the 8.3S cDNA was incubated with its 14S mRNA template, the bouyant density centrifugation profile (Fig. 3D) indicated a comparable density shift. In the latter case, besides the major RNA-DNA hybrid peak, additional minor density populations were also observed. In comparable experiments, rabbit globin RNA and QB RNA only gave trace amounts of hybrid products.

Discussion: Consistent with the results obtained by other investigators with RNA-dependent DNA polymerase (10,11), RNase A alone abolished virtually all the template activity. In comparison to other systems (11), the addition of actinomycin D inhibited about 50% of the net ^{3}H-dTMP incorporation. The latter result indicates a likely suppression of double-stranded DNA synthesis. Nevertheless, it should be noted that RNA-dependent synthesis of single-stranded DNA would not be inhibited by actinomycin D (18,19).

The formation of a 7.6S cDNA product suggests that there was substantial transcription of the 10S mRNA template. This observation is similar to those

obtained with globin mRNA (9,10). However, with the lens 14S mRNA, a considerably smaller portion of the nucleotide chain was transcribed since only an 8.3S cDNA was observed. Such size relationships in the transcription data are similar to those found in the translation data. The 10S mRNA codes for alpha crystallin B chains, molecular weight of 22,500, while the 14S mRNA is translated into A chain polypeptides, molecular weight of 19,500 (14,15,20). It is not clear why the 14S messenger molecule is translated and transcribed to a lesser extent.

The hybridization data clearly indicate a close homology between the cDNAs synthesized and their mRNA template counterparts. Control hybridizations with QB and rabbit globin RNAs suggest essentially no homology.

Messenger RNA templates, transcribed by AMV RNA-dependent DNA polymerase have been shown to contain poly(rA) segments (9,10,11,21). Recent experiments with lens mRNA indicate that these species also contain adenosine-rich clusters (22). Since little AMV RNA-dependent DNA polymerase activity was found with polysomal RNA obtained from lenses incubated with cordycepin in organ cultures, the presence of adenosine-rich clusters may be necessary for reverse transcriptase activity (22).

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