Synthesis of DNA complementary to the mRNAs for milk proteins by E.coli DNA polymerase I.

Louis-Marie Houdebine

Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, 78350 - Jouy-en-Josas, France.

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SUMMARY

E.Coli DNA polymerase I (Klenow subfragment) was used for the synthesis of complementary DNA with the mRNAs for rabbit milk proteins as templates. The cDNA formed, contained 200 nucleotides and represented about 20 % of the mRNA template. The cDNA was hybridized specifically to the mRNA templates. The Klenow subfragment of the E.Coli DNA polymerase I was as efficient as the avian myeloblastosis virus reverse transcriptase in the synthesis of cDNA. The mean size of the cDNA fragments obtained with the Klenow enzyme proved to be 70 % of the value obtained with the AMV reverse transcriptase and at least twice the value generally obtained with the complete E.Coli DNA polymerase I. The cDNA was used for the detection and the quantification of the mRNA template in various RNA fractions.

INTRODUCTION

Recent works demonstrated that the DNA obtained by reverse transcription from various RNA templates could be used to detect and quantify specifically the sequence of the template. This included the titration of specific mRNAs [1,2], the quantification of genes corresponding to various proteins [3], the identification of mRNA sequence in the RNA [4] and the sequencing of mRNA [15,16]. In most of these works, reverse transcriptases of viral origin were employed for the synthesis of cDNA.

Recent studies revealed that E.Coli DNA polymerase I exhibited a high activity for the synthesis of DNA from various RNA templates. cDNAs able to hybridize specifically to their templates were synthesized from 28 S, 18 S and 5 S rRNAs [5,6,10], mRNAs [13,7,14,9,15,16,17] and viral RNAs [13,12,11,8]. However, the cDNAs synthesized with the E.Coli DNA polymerase were generally much smaller than with the viral reverse transcriptase.

In the present study, the Klenow subfragment of the

E.Coli DNA polymerase I from which the 5' - 3' exonuclease was removed through proteolysis, was used for the synthesis of DNA complementary to the mRNAs for milk proteins. This enzyme was capable of synthesizing cDNA fragments of at least twice the length of those generally obtained with the complete E.Coli DNA polymerase I. This cDNA was hybridizable specifically and quantitatively to the mRNA template.

MATERIALS AND METHODS

1) Preparation of the mRNAs

mRNAs for milk proteins were prepared by poly U-Sepharose chromatography from total polysomes of lactating rabbit as previously described [18].

2) Preparation of polysomal RNA with and without poly A

Polysomal RNA from lactating rabbit mammary gland was extracted by the phenol method at pH 9 as previously described[18]. This RNA fraction contained mRNA for caseins with and without poly A [19]. An aliquot of the polysomal RNA was chromatographed on poly U-Sepharose. The fraction not retained by the column contained the ribosomal RNA and the mRNAs without poly A.

Alternatively, the polysomal fraction was subjected to poly U-Sepharose chromatography without prior extraction by phenol as suggested by Pemberton et al.[20]. Polysomes were dissolved in TNES buffer. This fraction contained the ribosomal proteins, the ribosomal RNA and the mRNAs with and without poly A. The effluent from the poly U-Sepharose column contained the same components minus the mRNAs with poly A. The poly A-containing mRNA which was eluted from the column by lowering the ionic strength, exhibited an electrophoregram similar to that previously obtained after phenol extraction [21]. The yield of mRNA was about 10 % higher than after phenol extraction.

Abreviations used :

- TNES buffer : 0.01 M Tris - HCl pH 7.6, 0.12 M Na Cl, 0.1 mM EDTA, 0.5 % SDS
- TES buffer : 0.01 M Tris - HCl pH 7.6, 0.1 mM EDTA, 0.1 % SDS
- SDS : sodium dodecylsulfate

3) Synthesis of cDNA

cDNA was synthesized in a mixture containing : 50 mM Tris HCl pH 7.6, 1 mM Mn Cl_2, 50 mM KCl, 0.1 % β-mercaptoethanol, 50 µg/ml BSA, 5 % glycerol, 100 µM dCTP, dATP and dTTP, 0.5-50 µCi/ml ^3H - dGTP (10 Ci/m mole NEN) 1 A_{260} mRNA/ml, 0.2 A_{260} oligo T_{12-18}/ml, (Collaboration Research), E.Coli DNA polymerase I (Klenow subfragment) 1-20 units/ml (Boehringer) or AMV reverse transcriptase kindly supplied by Dr J. Beard N.I.H. After two hours at 37°C, the mixture was incubated overnight at 37°C with 0.3 M NaOH and neutralized by acetic acid. The cDNA was then precipitated by EtOH with denatured salmon sperm DNA as carrier. The cDNA was separated from nucleotides by filtration on Sephadex G-50.

For kinetic studies, 5-10 µl aliquots were taken from the incubates at different times. The cDNA was precipitated by TCA containing sodium pyrophosphate with RNA carrier. The precipitates were collected on glass fiber filters and the radioactivity estimated in a Packard scintillation counter.

4) Estimation of the cDNA molecular weight by sucrose gradients

At the end of the incubation an aliquot of the incubate was layered over a 5-20 % sucrose gradient in TNES buffer. Another aliquot was treated by 0.3 M NaOH for one hour at 37°C then layered over a 5-20 % sucrose gradient in 0.9 M NaCl, 0.1 M NaOH according to Studier [22]. Gradients were run at 24 000 rpm for 22 hours at 20°C in SW 25 rotor. Radioactivity of the fractions (2 ml per fraction) was estimated after precipitation by TCA and filtration on glass fiber filters. BSA, anti- α_s- casein purified by immunoadsorption [23] and tRNA used as markers, were centrifuged on parallel neutral sucrose gradients.

5) Estimation of the cDNA molecular weight by Sepharose-4B

After alkaline treatment and neutralization, aliquots of the incubates were fractionated with a Sepharose-4B column (0.6 cm diameter and 25 cm high) equilibrated with TES buffer. Radioactivity of the fractions (1 ml per fraction) was estimated by TCA precipitation and filtration on glass fiber filters. Chromatography of mRNA and tRNA used as markers was carried out

separately under the same conditions.

6) Estimation of the cDNA molecular weight by polyacrylamide gel electrophoresis

After alkaline treatment and neutralization, the cDNA was subjected to electrophoresis on 2.3 - 13 % polyacrylamide gel, as previously described [18]. After 3 hours at 80 volts, the gel was cut into slices 0.5 cm long. The slices were incubated overnight in 0.5 % SDS at 50°C. Positions of the 28 S, 18 S rRNA and tRNA markers were determined on parallel gels.

7) Estimation of the cDNA molecular weight by denaturing polyacrylamide gel electrophoresis

After alkaline treatment and neutralization aliquots of the cDNAs synthesize with the bacterial and the viral enzymes were subjected to electrophoresis in formamide as described by Staynov et al.[33]. Gels were calibrated with tRNA,5 S rRNA and 9 S rabbit globin mRNA. At the end of the run, the gels were scanned and cut into slices 0.5 cm long. The radioactivity was estimated after an overnight incubation of the slices in 0.5 % SDS.

8) Purification of the S_1 nuclease

S_1 nuclease was purified from Aspergillus oryzae α- amylase as described by Vogt [24]. DEAE cellulose was the only purification step [25]. The S_1 nuclease activity was detected in the eluate by hydrolysis of denatured DNA. Aliquots of the fractions were incubated with 0.6 A_{260} DNA in 1.5 ml acetate buffer (25 mM potassium acetate pH 4.5, 0.1 mM $ZnCl_2$, 0.1 M NaCl). After 30' at 45°C, the non-hydrolysed DNA was precipited with 2 ml $HClO_4$. The activity of the S_1 nuclease was then determined by measurement of the U.V. absorbance at 260 nm of the $HClO_4$ supernatant.

9) RNA-cDNA hybridization

Hybridization were conducted in a final volume of 50 µl. The hybridization mixture contained 20 mM Tris HCl pH 6.8, 0.1 mM EDTA, 0.1 % SDS, either 0.45 M NaCl, 0.6 M NaCl or 0.45 M NaCl with 50 % formamide, about 2000 cpm cDNA and various amounts of RNA fractions. After 2 to 6 hours at 65°C, the non-hybridized cDNA was hydrolysed by S_1 nuclease in the acetate buffer (0.5 ml) at 37°C for 45 minutes in the presence

of 10 µg denatured DNA. The cDNA in the hybrid was then precipitated by TCA. It was determined that the amount of S_1 nuclease was sufficient to ensure a total hydrolysis of the single stranded DNA and was not inhibited by NaCl, SDS, EDTA, formamide or excess RNA.

RESULTS

1) Synthesis of cDNA

Kinetic studies of the cDNA synthesis by the Klenow DNA polymerase revealed that the incorporation of ^3H dGTP was dependent upon the amount of enzyme. The linearity was maintained longer with lower enzyme concentrations. Results of figure 1 indicated that the synthesis of cDNA increased with the amount of enzyme up to a plateau at 10 units per milliliter.

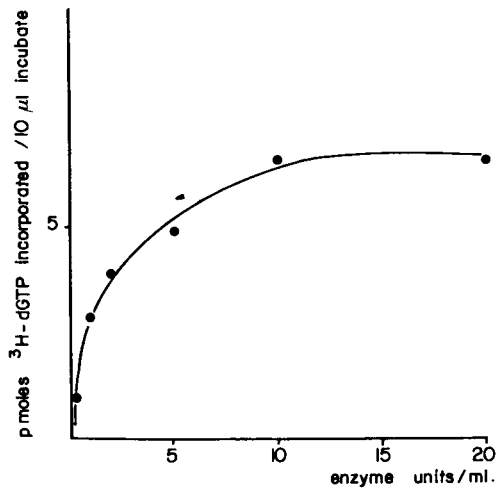

Figure 1 cDNA synthesis as a function of enzyme concentration. The incubation mixture contained in a final volume of 100 µl the same components as in figure 1, with variable amounts of enzyme (Klenow). The incorporation was measured by TCA precipitation in 10 µl aliquotes after 1 hour incubation.

The incorporation of ^3H dGTP was mRNA- and oligo T-dependent. The maximum activity was reached with 1.5 A_{260} mRNA per milliliter (with a constant ratio $\frac{A_{260}\ \text{mRNA}}{A_{260}\ \text{oligo T}} = 5$). Further additions of mRNA led to a lower synthesis possibly due to a relative insufficiency of enzyme [6] or to impurities in the mRNA which might have acted as inhibitors (figure 2).

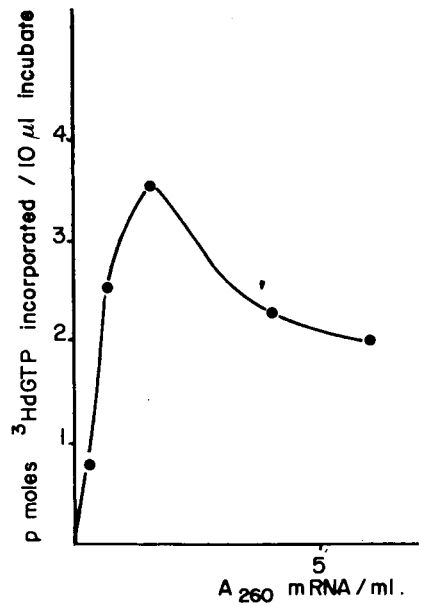

Figure 2 cDNA synthesis as a function of mRNA concentration. The conditions of incubation are the same as in figure 1. The ratio $\frac{\text{mRNA}}{\text{Oligo T}}$ = 5 was kept constant. The amount of the DNA polymerase (Klenow) was 0.6 unit per ml.

The cDNA synthesis was stimulated linearly by increasing amounts of labelled dGTP (from 10 to at least 50 µCi/ml incubate).

Addition of actinomycin D (100 µg/ml) slightly reduced the rate of the reaction. The addition of this antibiotic proved to be unnecessary since only a single stranded DNA was normally formed with the E.Coli DNA polymerase I [5].

2) Characterization of the cDNA

The molecular weight of the cDNA was estimated by four independent methods :

a) Centrifugation in alkaline sucrose (figure 3) indicated that the cDNA sedimented between BSA and purified anti-α_s- casein used as markers. In a neutral sucrose gradient and without prior treatment with alkali, cDNA had a sedimentation coefficient which is similar to RNA of 10 S.

b) Chromatography on Sepharose-4B showed that the major part of the cDNA after alkaline treatment was eluted before tRNA (figure 4). Successive chromatographies of cDNAs synthesized

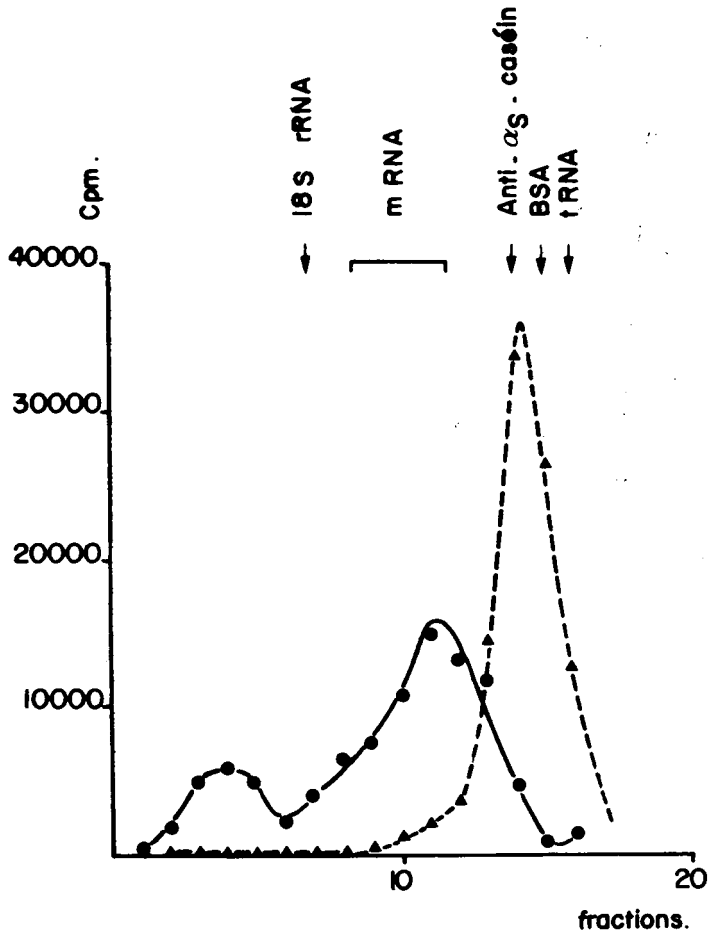

Figure 3 Sedimentation of cDNA in sucrose gradients. cDNA was synthesized with 1.5 A_{260} mRNA/ml and 10 units/ml Klenow subfragment of the E.Coli DNA polymerase I. After 1 hour incubation the cDNA was sedimented through sucrose gradients (see "Methods").

●——● neutral sucrose gradient

▲- - -▲ alkaline sucrose gradient

The arrows indicate the position of markers in neutral parallel gradients.

with various amounts of enzyme, demonstrated that the size of the cDNA was not dependent upon the enzyme concentration in the incubation mixture. cDNA prepared with AMV reverse transcriptase exhibited a similar molecular weight.

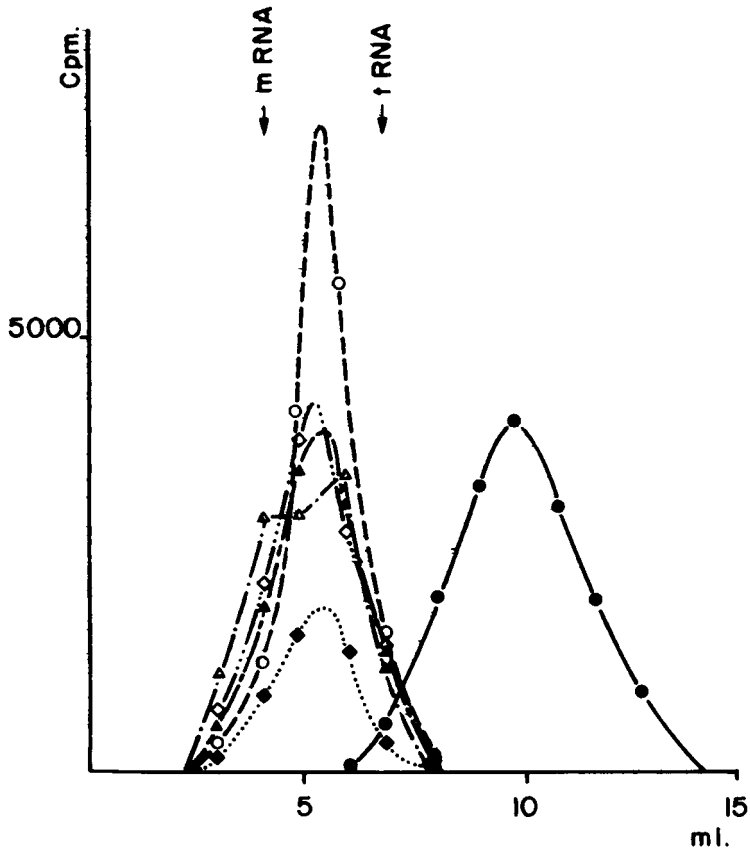

Figure 4 Chromatography of cDNA on Sepharose-4B. Radioactivity of fractions was determined by TCA precipitation as described in "Methods". cDNA was synthesized with :

o---o 0.6 unit/ml (Klenow [26,27,28])

◇-···-◇ 12.5 units/ml "

△—·—△ 25 units/ml "

◆·····◆ 10 units/ml " + unlabelled dGTP 50 µM

●——● DNA polymerase purified according to Richardson [29]

▲---▲ AMV reverse transcriptase

c) After treatment by NaOH, the cDNA migrated in a polyacrylamide gel as molecules longer than tRNA (figure 5).

d) Polyacrylamide gel electrophoresis in formamide led to a better estimation of the cDNA molecular weight (figure 6).

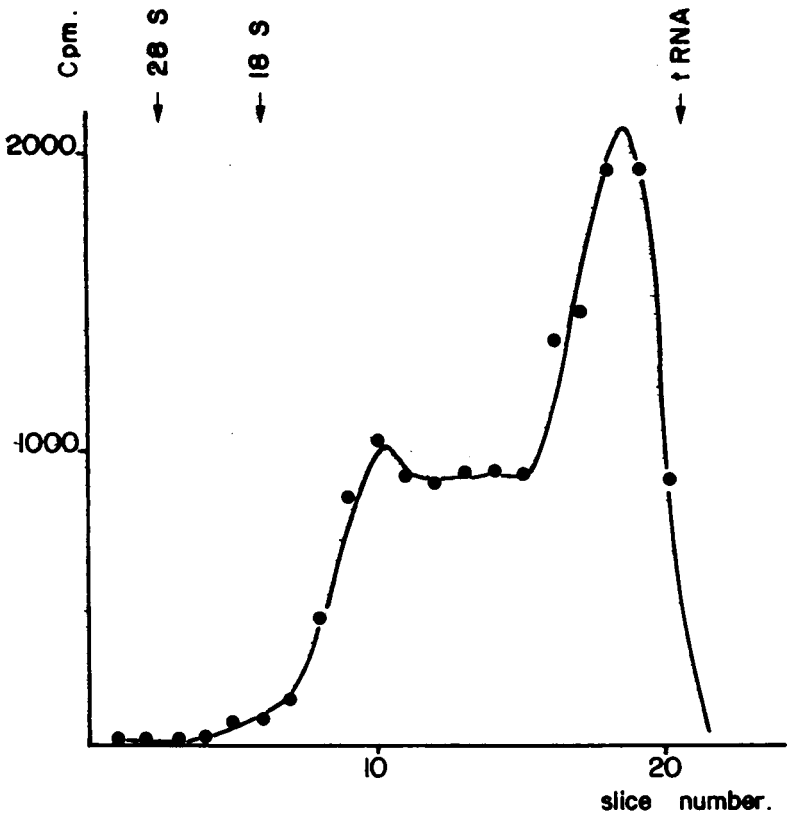

Figure 5 cDNA was synthesized with the Klenow subfragment of E.Coli DNA polymerase I (10 units/ml). After 3 hours incubation and alkaline treatment the cDNA was subjected to electrophoresis in polyacrylamide gel (see "Methods").
The arrows indicate the position of RNA markers in parallel gels.

The cDNA synthesized by the AMV reverse transcriptase contained an average nucleotides number of 300 with a broad distribution along the gel, in accordance with previous reports on DNA complementary to globin mRNA [34]. The cDNA synthesized by the Klenow enzyme contained 200 nucleotides with less heterogeneity in the fragments size. The same length was observed when purified casein mRNA [23] and rabbit globin mRNA were used as templates (data not shown).

All four methods used for determining the molecular weight of cDNA gave similar results. The cDNA synthesized with the Klenow subfragment [26,27,28] was significantly longer than

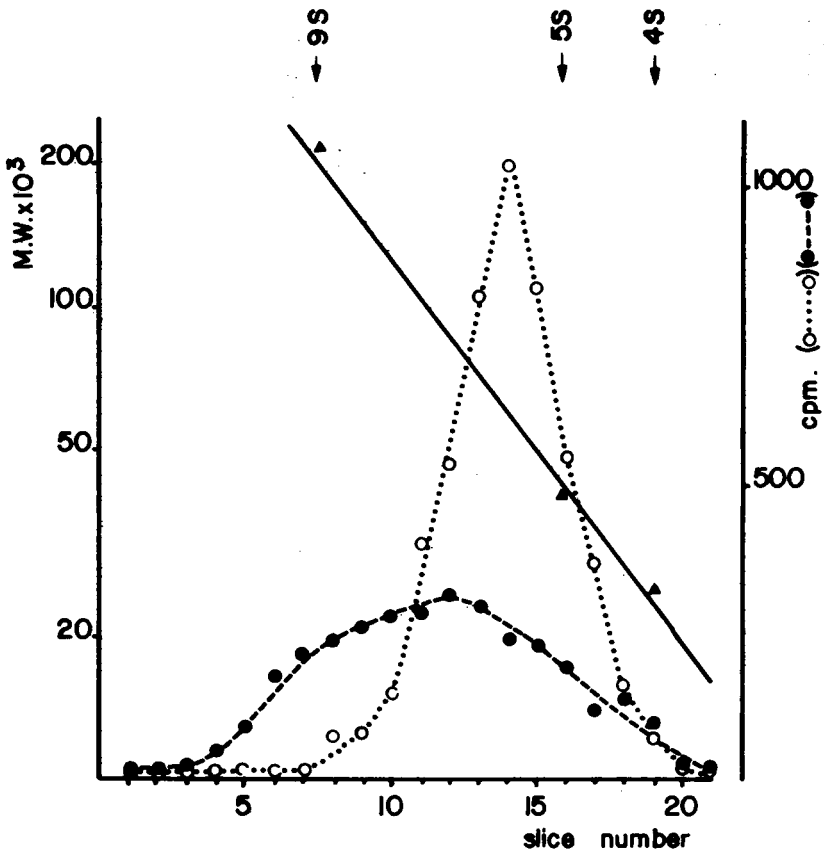

Figure 6 Polyacrylamide gel electrophoresis in formamide. Electrophoresis was conducted in 98 % formamide, 20 mM NaCl, 0.1 mM EDTA with 4 % acrylamide gels for 4 hours at 160 volts.
The arrows indicate the position of RNA markers in parallel gels.

o·····o cDNA synthesized with the Klenow enzyme

●----● cDNA synthesized with the AMV reverse transcriptase

with the complete E.Coli DNA polymerase I purified according to Richardson [29] (step IV) (figure 4).

Based upon the estimation of its molecular weight the cDNA synthesized by the Klenow enzyme contained 200 nucleotides which corresponds to about 20 % of the size of the mRNA template [18,21]. The length of this cDNA is therefore 70 % of that commonly obtained with the viral reverse transcriptase.

3) Hybridization of the cDNA with the mRNA template

After alkaline treatment and purification on Sephadex G-50, the cDNA was hybridized to various RNA fractions.

Hybridization of the cDNA was first carried out with increasing amounts of mRNA (purified by poly U-Sepharose) total polysomal RNA and with yeast tRNA. About 90 % of the cDNA was capable of hybridizing to the mRNA. The $Cr_0t\ \frac{1}{2}$ expressed in standard conditions (0.12 PB) proposed by Britten and Smith [31] was 9.10^{-2} moles $litre^{-1}$ second for the mRNA fraction and 6.5 moles $litre^{-1}$ second for the total polysomal RNA (figure 7). These values are compatible with the presence in the mRNA fraction of the 5 major mRNAs for milk proteins corresponding to 70-80 % of the mRNAs [21,23] (with about 1000 nucleotides in each mRNA). Moreover, the ratio $\frac{Cr_0t\ \frac{1}{2}\ \text{polysomal RNA}}{Cr_0t\ \frac{1}{2}\ \text{mRNA}} \simeq 70$ is in good agreement with the proportion of mRNA in polysomal RNA [18,19,21]. The hybridization with yeast tRNA was negligible. This indicates that the cDNA can form a specific hybrid only with the sequence of the mRNA template. When hybridization was carried out in buffer containing 0.45 M NaCl or 0.45 M NaCl - 50 % formamide, the reaction rate was slower but exhibited the same specificity (data not shown).

A hybridization experiment conducted simultaneously with the cDNAs obtained with the E.Coli polymerase and the AMV reverse transcriptase, revealed that both cDNAs were capable of hybridizing to the mRNA template with almost equal efficiency (figure 7).

Previous work in this laboratory demonstrated that a large fraction of the mRNAs for caseins was devoid of poly A-sequences [19]. Such mRNAs did not bind to poly U-Sepharose but were able to direct the synthesis of caseins in a reticulocyte lysate. A similar non poly A-containing mRNA was also found in the rat mammary gland [32]. The hybridization technique used above for the detection of the mRNAs in the polysomal fraction (figure 7) was used to quantify the mRNAs with and without poly A. The cDNA was therefore incubated with various quantities of total polysomal RNA before and after chromatography on poly U-Sepharose. Results of figure 8 indicated that the RNA fraction not retained by the column contained a significant amount of RNA

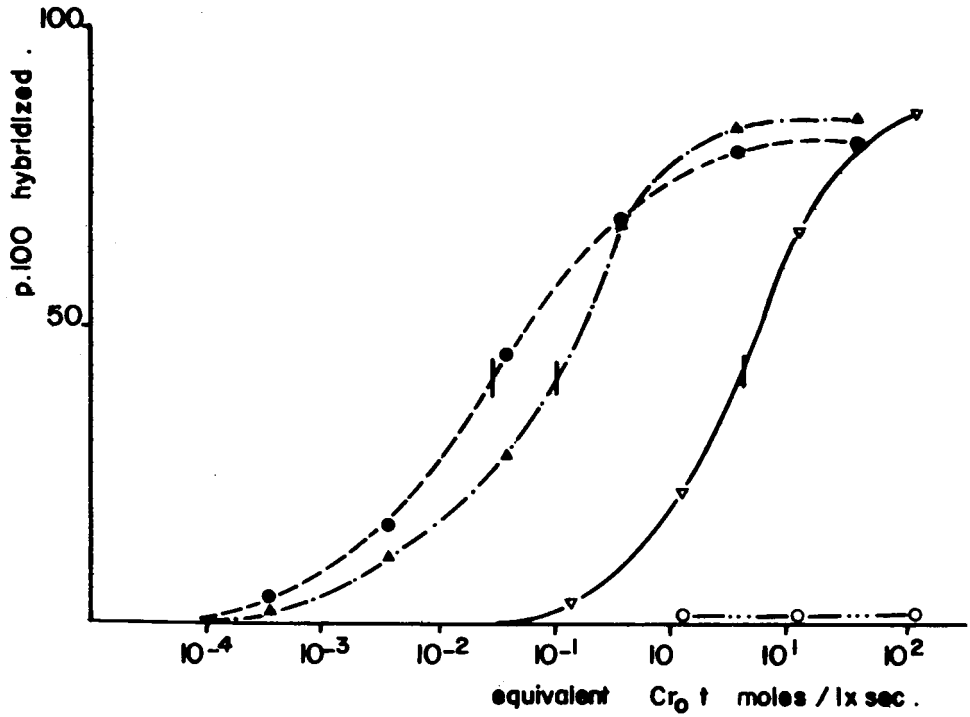

Figure 7 Hybridization kinetic curves of the mRNA template with the cDNA. Hybridization was conducted in buffer containing 0.6 M NaCl for 6 hours at 65°C. At the end of incubation, the non-hybridized cDNA was hydrolysed by S_1 nuclease (see "Methods"). Results are express in the standard conditions 0.12 PB of Britten and Smith [31].
(Cr_0t in moles/litre x seconde).

▲—·—▲ cDNA synthesized with the E.Coli polymerase I (Klenow) and hybridized with the mRNA template.

●– – –● cDNA synthesized with the AMV reverse transcriptase and hybridized with the mRNA template.

▽———▽ cDNA synthesized with the E.Coli polymerase I and hybridized with the total polysomal RNA.

○—···—○ cDNA synthesized with the E.Coli polymerase I and hybridized with yeast tRNA.

sequences identical to the poly A-containing mRNAs. When the hybridization was carried out with the RNA fractions obtained without prior phenol extraction (see "Methods"), the proportion of the mRNAs with and without poly A was similar (figure 8). These experiments indicate that this quantification of mRNA yields the same results as previously obtained by mRNA translation. Moreover these results confirm that the phenol extraction

step does not remove poly A-sequences from mRNA.

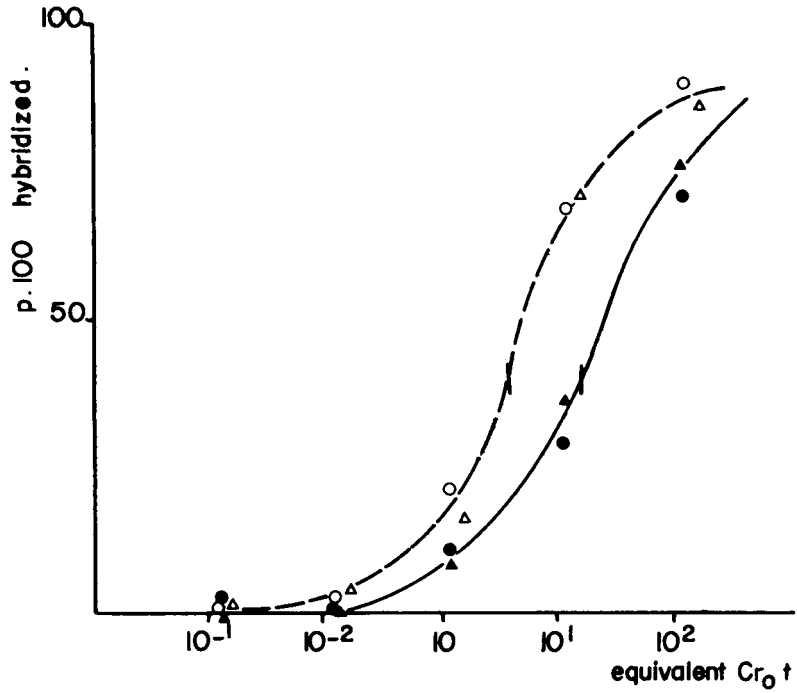

Figure 8 Hybridization kinetic curves of the cDNA with RNA fractions containing the mRNA template. Conditions of the reaction were that of figure 7.

o---o total polysomal RNA after phenol extraction.

Δ---Δ total polysomal RNA without phenol extraction (see "Methods")

●——● total polysomal RNA after phenol extraction and chromatography through poly U-Sepharose.

▲——▲ total polysomal RNA without phenol extraction and after chromatography through poly U-Sepharose.

CONCLUSIONS

The present work demonstrates that the Klenow subfragment of the E.Coli DNA polymerase I could direct the synthesis of DNA complementary to the mRNAs for milk proteins. The molecular weight of this cDNA estimated by four independent techniques corresponds to a polynucleotide consisting of 200 bases. This enzyme is thus able to copy about 20 % of the mRNA template. The Klenow polymerase was as active as the AMV reverse transcriptase although the mean size of the cDNA was lower with the

bacterial enzyme. The DNA polymerase I purified according to Jovin et al.[30] (step VII) was also efficient for reverse transcription but the resulting cDNA generally did not contain more than 100 bases [5,6,7,8,9,11,13,15,16,17]. The same E.Coli polymerase I purified according to Richardson (step IV) [29] led to the synthesis of very short cDNAs which cannot be used for hybridization. This is probably due to contaminating nucleolytic activities.

The cDNA was hybridizable specifically and quantitatively to the mRNA template. This cDNA could be used to quantify the mRNA template in various RNA fractions, in agreement with previous results obtained by translational techniques. However, the cDNA synthesized with the E.Coli DNA polymerase I, compared to the cDNA obtained with the viral enzyme, exhibited a slightly lower efficiency of hybridization. This difference was not observed by Gulati et al.[13] who used the enzyme purified according to Jovin [30]. However, these authors mentioned that the hybrid formed with the cDNA and the mRNA template melted at a lower temperature (3°C lower) when the cDNA was synthesized with the E.Coli enzyme. The slightly lower hybridization rate observed with the bacterial enzyme may be attributed to the smaller size of the cDNA fragments or to a less faithfull copy of the mRNA template. The specificity of the cDNA toward the mRNA for hybridization is obviously not altered by the possible resulting mismatching.

The Klenow subfragment of the E.Coli DNA polymerase I may thus be considered as a viable substitute for the AMV reverse transcriptase for the reverse transcription of mRNAs. The greater availability of the Klenow enzyme makes it a precious tool for mRNA-cDNA hybridization studies.

Recent work in this laboratory pointed out that prolactin induces the synthesis of the mRNAs for caseins titrated by translation and that progesterone prevents this induction [18]. A new evaluation of these results is now in progress by using DNA complementary to mRNAs for caseins purified by immunoprecipitation of the polysomes synthesizing these proteins [23].

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