SYNTHESIS OF PROBES FOR RNA USING Qβ-REPLICASE

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SUMMARY: Qβ replicase was used to make labeled RNA complementary to 5S, 18S and 28S ribosomal RNA and 9S histone mRNA. These copies appear to be the same size as the parental RNA strand. The kinetics of annealing of these probes shows a proportionality between the rate and the complexity of the RNA molecule used as template.

INTRODUCTION

The discovery of RNA dependent DNA polymerase, reverse transcriptase, provided a powerful new method for quantitation of specific RNA molecules. Specific cDNA probes can be synthesized using this enzyme, a template polyadenylated RNA, and an oligo-dT primer (1,2,3). The cDNA can then be used as a highly specific probe to quantitate the RNA used as template in a complex mixture of RNA. However, the method is not applicable to non-polyadenylated RNA unless a primer complementary to the 3' end is available or polyA is enzymatically added to the 3' end (4,5).

The RNA replicase induced as a result of infection of E. coli by Qβ bacteriophage is normally highly specific for its parental RNA genome (6). However, addition of Mn^{++} to the reaction mixture relaxes this specificity and promotes copying of other unrelated RNA's (7). This reaction can be used to synthesize RNA probes for non-polyadenylated RNA such as 5S, 18S, 28S and histone mRNA.

MATERIALS AND METHODS

Purification of Qβ-Replicase. Qβ phage mutant amB86 and E. coli strains K37 and A19 were kindly supplied by Dr. A. Palmenberg. The replicase was purified by the method of Kamen (8). 50 g of infected cells yielded about 4 mg of purified enzyme.

Isolation of RNA. HeLa 18S and 28S RNA were isolated by sucrose gradient centrifugation. HeLa 5S RNA was purified by polyacrylamide electrophoresis. Qβ RNA was

prepared by phenol extraction of virus purified by CsCl density gradient centrifugation. Chick ovalbumin mRNA was a gift of Drs. Savio Woo and Bert O'Malley.

The procedure for preparation of HeLa or mouse L-cell 9S histone mRNA was essentially that of Breindl and Gallwitz (9). The cells were synthesized by a double thymidine block. Polysomal RNA was prepared from early S phase cells, loaded onto a sucrose gradient and centrifuged at 39,000 rpm for 24 hours at 4° in a SW40 rotor. The 9S region was recovered, twice re-run on sucrose gradients and further purified by electrophoresis on a 6% polyacrylamide gel. The 9S RNA was recovered from the gel by electrophoresis. Drosophila 9S RNA was prepared by similar methods from unsynchronized Schneider's line 2 cells grown in culture.

Synthesis of RNA using Qβ-Replicase. Conditions of synthesis were those in Palmenberg and Kaesberg (7). The incubation mixture, 0.5 ml contained 80 mM Tris, pH 7.5, 12 mM β-mercaptoethanol, 12 mM $MgCl_2$, 1 mM $MnCl_2$, 0.8 mM GTP, ATP and CTP, 0.08 mM UTP, 20 μCi ^{3}H-UTP (15 Ci/mmole), 1 or 2 μg of template RNA and 10 μg of Qβ replicase. Incubations were normally for 30 min at 31°. The reaction was terminated by addition of EDTA, 50 mM and SDS, 0.5%. RNA was extracted with phenol and purified by Sephadex G-50 gel filtration.

Polyacrylamide Gel Electrophoresis of Product RNA. Product RNA was denatured by boiling in 6 M urea and electrophoresed in 3% or 6% gels containing 6 M urea (10).

Renaturation Kinetics of RNA. Purified product RNA was sealed in capillaries in 6xSSC, 45% formamide, denatured by boiling, and incubated at 50°. At appropriate times the contents of the capillaries were diluted into 1 ml of 2xSSC, treated with ribonuclease, 5 μg/ml, for 30 min at 37° and TCA precipitated in the presence of yeast carrier RNA.

RESULTS

Synthesis of Complementary RNA Using Qβ Replicase

When only magnesium is present as a co-factor, Qβ replicase uses only Qβ RNA, plus and minus strands, synthetic polymers such as polyC or Qβ specific 6S RNA as template (11,12). It has been suggested that the 6S RNA can occur as a tightly bound contaminant of purified Qβ replicase (13), thus the enzyme will not demon-

TABLE 1

Time (min)	RNA Template (nM UMP Incorporated) --	Drosophila 9S	Qβ	HeLa 28S
10	0.08	.6	1.8	.55
20	0.17	1.6	5.1	1.4
30	0.8	5.4	7.2	2.1
40	5.6	8.0	8.7	2.0
50	8.5	9.6	12.4	2.9
60	9.4	11.8	15.5	3.5

Each template RNA was present at 10 µg/ml in a total volume of 100 µl. Incubation was at 31°. Samples were taken and the reaction terminated by dilution and subsequent precipitation with TCA at the indicated times.

strate complete template dependence, particularly after prolonged incubation periods during which this trace contaminant is amplified. Other recent work (14) suggests the possibility that Qβ replicase is capable of synthesizing RNA independent of any template. Examples of template dependence after short incubations and the loss of this dependence are shown in Table 1. The period of incubation during which strict template dependence holds appears to vary from preparation to preparation of the enzyme.

Even when manganese as well as magnesium is incorporated into the reaction mixture, Qβ RNA is the preferred template for Qβ replicase. A series of different RNA's available to us were used as templates in parallel reactions and the results are listed in Table 2.

It has previously been reported that the copies of heterologous RNA made by Qβ replicase are approximately the same length as the parental strand (7). This observation is confirmed by the gel electrophoresis patterns of the products made from HeLa 18S and 9S histone mRNA, Figure 1. In each case the sample was heat denatured in 6 M urea and run under denaturing conditions (10).

TABLE 2

Template	Relative Template Activity
Qβ	100
HeLa 18S	49
HeLa 28S	30
Drosophila 9S	62
Mouse 9S	64
Ovalbumin mRNA	69
None	5

Template concentration for all reactions was 10 µg/ml. The reaction was incubated for 15 min at 31° and terminated by dilution and subsequent precipitation with TCA.

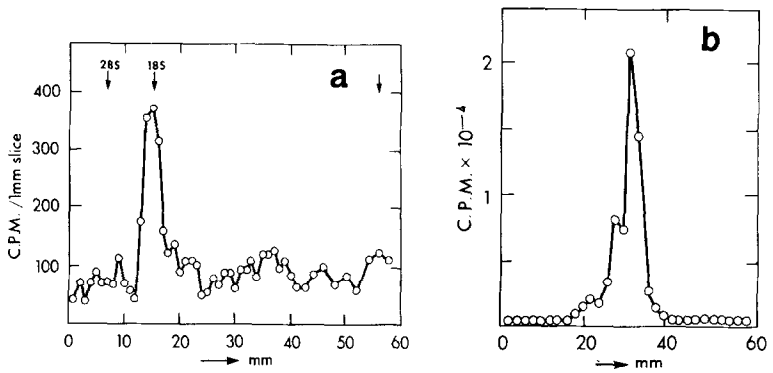

Figure 1. Polyacrylamide gel analysis of product RNA. (a) ^{3}H-labeled RNA synthesized using HeLa 18S RNA as template. Electrophoresis in a 3% gel containing 6 M urea; (b) ^{3}H-labeled RNA synthesized using HeLa 9S RNA as template. Electrophoresis in a 6% gel containing 6 M urea. In each case, the RNA sample was denatured by boiling in 6 M urea.

Renaturation of Product RNA

After synthesis of the complementary strand, RNA was prepared from reaction mixtures containing HeLa 5S, 18S and 28S RNA and self-annealed. The con-

ditions 6xSSC, 45% formamide and 50° were chosen to account for the high Tm of double stranded RNA. The kinetics of renaturation are illustrated in Figure 2. Self-annealing of the 5S RNA product is much more rapid than that of 18S and 28S in accordance with the relative molecular weights of the three RNA species.

Just as the case for renaturation of DNA, a proportionality is expected between the $(RR)_o t_{1/2}$ value, representing 50% renaturation and the molecular weight of the RNA. This expectation is fulfilled since a straight line relationship is obtained when these two parameters are plotted for four RNA templates of known molecular weights (Fig. 3).

Similar renaturation kinetics were established for replicase products made from HeLa or Drosophila 9S RNA and Qβ RNA (Fig. 4). In both cases the 9S mRNA specific RNA anneals at a rate approximately twice that for Qβ RNA with a $(RR)_o t_{1/2}$ value of approximately 4×10^{-3} suggesting an analytical complexity of about 7×10^5 daltons (Fig. 3).

DISCUSSION

Production of cDNA probes for annealing kinetics has proved to be a powerful

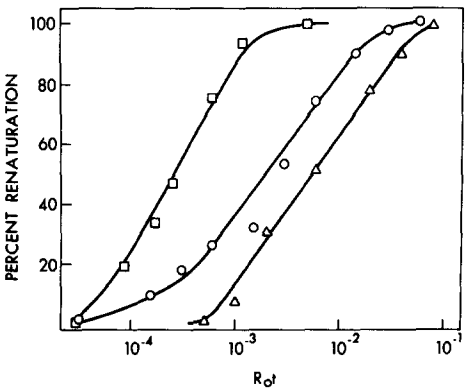

Figure 2. Renaturation kinetics of product RNA. ^{3}H-RNA synthesized using HeLa 5S, 18S and 28S RNA as templates were denatured and annealed in 6xSSC, 45% formamide at 50°. The curves from left to right represent the renaturation kinetics of 5S, 18S and 28S RNA, respectively. Background levels of double-stranded RNA at zero time were subtracted in each case: 5S RNA, 33%; 18S RNA, 20%; 28S RNA, 25%.

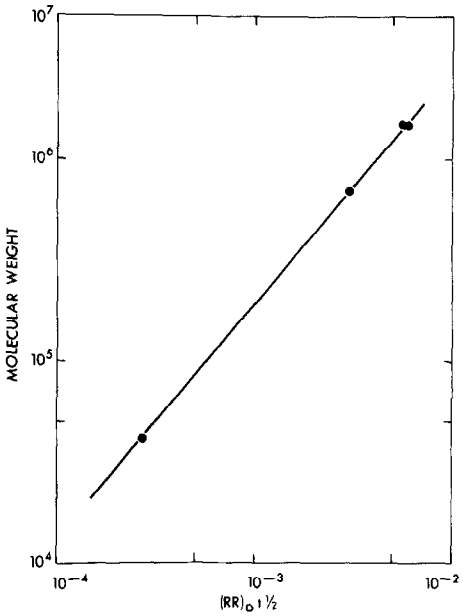

Figure 3. The relationship between the kinetics of self-annealing of the product and the molecular weight of the template RNA. The $(RR)_o t_{1/2}$ values from Fig. 2 are plotted against the molecular weights of the three template RNA's. The fourth point represents the value for Qβ RNA taken from Fig. 4. The molecular weights are taken to be 5S RNA, 4×10^4, 18S RNA, 0.7×10^6; 28S RNA, 1.5×10^6; Qβ RNA, 1.5×10^6.

approach for quantitating various messenger RNA's. However, not all RNA molecules of interest are polyadenylated making this same methodology difficult to apply. The present preliminary data suggest that Qβ replicase may be used to synthesize probes for RNA. However, the capacity of the enzyme to synthesize RNA without added template, either de novo or because of Qβ specific 6S RNA contamination, cuases serious problems in the use of this enzyme for synthesis of probes. However, under conditions of template dependence, the molecular weight of the RNA produced approximates that of the template as demonstrated earlier by Palmenberg and Kaesberg (7).

Complementary RNA probes may be used to estimate the analytical complexity and therefore purity of the RNA used as template, in a manner analogous to the annealing of cDNA with its template RNA (1,2,3). In this case the $R_o t_{1/2}$ value for annealing is directly proportional to the complexity of the template RNA (15).

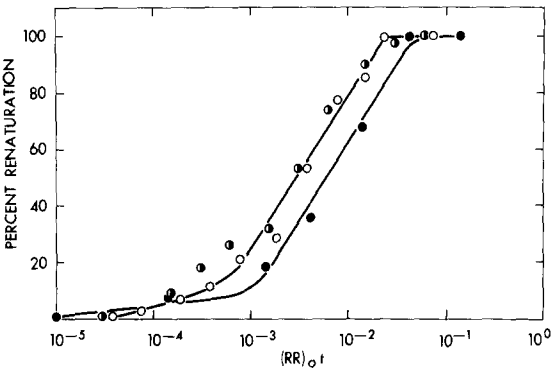

Figure 4. Renaturation kinetics of ^{3}H-RNA synthesized using Qβ RNA (●---●), HeLa 9S RNA (O---O) and Drosophila 9S RNA (◑---◑) as template. Conditions as in Fig. 2.

We have attempted to validate this principle in the case of RNA/RNA reactions by examining the self-annealing kinetics of various RNA's copied with Qβ replicase. To a first approximation the rate of annealing is proportional to molecular weight of the template RNA. Further work will be necessary to examine other parameters such as the size of the RNA in the annealing reaction and the optimal conditions for reaction.

The non-polyadenylated 9S RNA of sea urchins and HeLa cells appears to represent the mRNA for the five species of histone (9,16). These five species of RNA have molecular weights of approximately 1.4 x 10^5 daltons (17). Therefore the expected complexity of the group of mRNA's is approximately 7 x 10^5 daltons. When the kinetics of annealing of a copy of HeLa or Drosophila 9S RNA with its template were measured, the rate of reaction appeared to be approximately twice that for a corresponding annealing mixture made up of Qβ RNA and its replicase copy. This result is consistent with a high degree of purity for these two preparations of histone mRNA.

Again by analogy to current exploitation of cDNA in hybridization assays, it is apparent that replicase copies could be used to quantitate particular species of RNA in a complex mixture. In the case of RNA probes, it is necessary

to separate the product strand from the template strand. This could probably be accomplished by tethering the template strand to a solid support or by exploiting differences in size or base composition. In the particular case of histone mRNA we are attempting to develop such methodology in order to quantitate *in vitro* transcription of histone genes.

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