SYNTHESIS OF RNA COMPLEMENTARY TO RABBIT GLOBIN mRNA by Qβ REPLICASE*

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SUMMARY: RNA, complementary to rabbit globin messenger RNA, is synthesized by Qβ replicase in a reaction primed by either oligo(rU) or oligo(dT). The radioactive product is in a double-stranded hybrid form with its mRNA template following synthesis. The reaction generates nearly full-length copies of the mRNA, and a series of discrete length partial transcripts. In addition, non-specific RNA between 90-140 nucleotides in length is produced.

The Qβ replicase, a four-subunit enzyme induced as a result of RNA phage Qβ infection of Escherichia coli (1, 2) is an RNA-dependent RNA polymerase highly specific for Qβ RNA among all natural RNAs tested (3,4). This enzyme will also copy poly(rC) and other polyribonucleotides rich in cytidylate (5). The extreme template specificity of the replicase for Qβ RNA can be relaxed in two ways. First, when Mn^{++} as well as Mg^{++} is added to the reaction mixture, the enzyme will accept several natural RNAs as templates (6, 7, 8). The product of this reaction is nearly full-length RNA, annealed to its template, and has ppG as the 5'-terminal nucleotide in all cases (7). Qβ replicase is only about 10% as active in the presence of Mn^{++} as it is in the absence of Mn^{++} when Qβ RNA is used as template (6, 7). Second, it has recently been observed that high concentrations of oligonucleotides

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complementary to sequences found in the template allow the enzyme to bypass its normal initiation specificity, and extend complementary synthesis from the 3' end of the oligonucleotide primer (9). Unlike the Mn^{++} reaction, the oligonucleotide-primed reaction does not reduce the efficiency of the replicase, and indeed may enhance it. In this paper we show that Qβ replicase can transcribe rabbit globin messenger RNA efficiently, when the reaction is primed with either oligo(rU) or with oligo(dT), that the product may be separated from the template by oligo(dT)-cellulose chromatography, and that full-length transcripts and partial transcripts of discrete length are synthesized, along with nonspecific RNA, 90-140 nucleotides long.

MATERIALS AND METHODS

Purification of Qβ replicase. Frozen Qβ am B860 infected E. coli mx 248 cells were obtained from Dr. T. Blumenthal. Replicase was purified to homogeneity by the method of Kamen (10) with modifications as outlined by Blumenthal, et al., (11).

Isolation of globin mRNA. Rabbit globin mRNA was isolated from whole red blood cells of anemic rabbits as described by Nienhuis, et al. (12), with minor modifications. Total RNA was passed over an oligo(dT)-cellulose (Searle) column, and further purified by sucrose gradient centrifugation and a second oligo(dT)-cellulose column, as described (13). Globin mRNA obtained in this way was radio-iodinated as described (14), and migrated as one band in a 5% polyacrylamide - 98% formamide gel (see figure 1, slot 1).

Transcription of mRNA by Qβ replicase. The standard reaction consists of a 50 µl mixture of the following components: 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA-Na_2, 20 µg/ml globin mRNA, 60 µg/ml primer ($oligo(rU)_{6-8}$ or $oligo(dT)_{12-18}$, Collaborative Research), 60 µg/ml Qβ replicase, and 0.5 mM ATP, GTP, UTP and CTP, respectively. Reactions contained either [^{32}P]-NTP's (all four present in equal concentrations; final specific activity, 0.3-1.0 x 10^3 cpm/pmol; New England Nuclear) or [^{14}C]-ATP (specific activity, 600 mCi/mmol; Amersham Ltd). Reaction mixtures were heated at 80°C for 1 minute and were quick-cooled on ice prior to the addition of enzyme. They were then incubated for up to two hours at 35°C. Aliquots were removed at different times and were analyzed for trichloroacetic acid-precipitable radioactivity. Reaction products were phenol extracted, applied to Sephadex G-150 columns (0.7 x 30 cm), then were eluted with sterile, deionized water. The excluded material was adjusted to 0.10 M sodium acetate (pH 5.0), and 2.5 volumes of 95% ethanol was added along with carrier transfer RNA to precipitate RNA products.

RNA electrophoresis. Precipitated RNA was dissolved in 10 µl deionized formamide and marker dyes were added. Samples were heated at 80°C for 1 minute, rapidly cooled on ice and electrophoresed in 5% polyacrylamide-98% formamide slab gels, as described (15).

Enzyme digestion. Aliquots of the reaction mixture were digested directly with RNase A + T1 (Worthington), or with S1 nuclease (purified as described (16)) using the conditions described in Table 2.

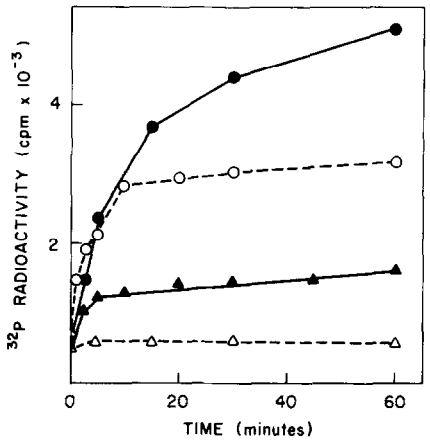

Figure 1. Yield of [^{32}P]-RNA synthesized as a function of time. One µl aliquots of a 50 µl standard reaction mixture, primed with oligo(rU) (●—●—● , total cpm 1.38 x 10^7), primed with oligo(dT) (○-○—○ , total cpm 1.64 x 10^7), containing oligo(rU) but no mRNA template (▲ - ▲ - ▲, total cpm 2.07 x 10^7), or containing oligu(rU) and mRNA but no enzyme (Δ - Δ - Δ, total cpm 1.22 x 10^7) were assayed by trichloroacetic acid precipitation. Each point corresponds to an average of duplicates. Data is normalized to the same total cpm as the oligo(rU) primed reaction for proper comparison.

Oligo(dT)-cellulose chromatography. For the separation of the labeled product from the unlabeled template, Sephadex purified and ethanol precipitated double-stranded RNA was dissolved in 85% formamide and was heated at 75°C for 3 minutes. It was then diluted 20-fold with binding buffer (0.5 M KCl, 0.01 M Tris-HCl (pH 7.5)) and passed over oligo(dT)-cellulose. The non-bound fraction, containing 86% of the labeled material, was precipitated by addition of 2.5 volumes of 95% ethanol.

RESULTS AND DISCUSSION

Synthesis of cRNA by Qβ replicase. The kinetics of the incorporation of [^{32}P]-nucleoside triphosphates into RNA by Qβ replicase in reactions utilizing rabbit globin mRNA as template and either oligo(rU) or oligo(dT) are shown in figure 1. The data have been normalized to the same total cpm per reaction mixture. The oligo(rU) primed reaction continues beyond one hour, whereas the oligo(dT) primed one levels off after approximately 30 minutes. Considerable amounts of acid precipitable material are generated when the reaction contains all components except the globin mRNA template. No reaction is observed in the absence of enzyme, although

TABLE 1. cRNA Synthesis by QB replicase using Rabbit Globin mRNA as Template

Reaction Conditions	pmoles NMP incorporated in 60 minutes*
Oligo(rU) primed:	
Complete	365,323
+ 0.10 MNaCl	33
- enzyme	41
- template	117
Oligo(dT) primed:	
Complete	229,265, (255)
- template	(97)
- enzyme	(35)

* Each number in the table represents a separate experiment. All values were obtained using [^{32}P]-labeled NTP precursors, except those in parentheses, which resulted from experiments using [^{14}C-ATP].

a consistently high background appears at all time points. These counts represent between 10-15% of the total CPM incorporated into RNA in one hour by the template-primer directed reactions, and as much as 40% of the counts obtained in the absence of template mRNA. We observed similar results when [^{14}C]-ATP was used as the source of radioactivity. The total number of picomoles of nucleoside-monophosphate incorporated into RNA per 50 µl reaction mixture is summarized in Table 1 for several individual experiments. The reaction is completely inhibited by the addition of 0.10 M NaCl.

Fidelity of transcription by Qβ replicase. The extent of the production of RNA complementary to rabbit globin mRNA by the template-primer dependent Qβ replicase reaction was assessed as follows. Samples of a complete reaction mixture with oligo(rU) as primer were phenol extracted after two hours of incubation, and passed over sephadex. They were precipitated by the addition of 95% ethanol, redissolved in the appropriate buffer, and

TABLE 2. S1 Nuclease and RNase A + T1 Resistance of Transcription Product

	% S1 Resistance	% RNase A + T1 Resistance
No Incubation*	66.7	66.5
After Incubation**	74.1	73.3
After oligo(dT)-Cellulose Chromatography†	37	----

* An aliquot of the reaction mixture was digested for 45 minutes at 37°C with S1 nuclease, or RNase A + T1. The S1 nuclease reaction mixture consisted of: 0.2 M NaCl, 0.05 M Na Acetate (pH 4.5), 50 µg/ml sonicated and denatured DNA and 3 units (17) S1 nuclease. The RNase A + T1 reaction mixtures contained: 2 X PIPES buffer (10 X PIPES buffer is 1.75 M NaCl, 0.1 M PIPES, pH 6.7), and 230 µg/ml each RNase A and T1.

** An aliquot of the reaction mixture was made to 5 X PIPES buffer and incubated to a calculated Rot of 0.1. It was then digested with the nucleases as above.

† An aliquot of the reaction mixture was passed over oligo(dT)-cellulose chromatography (see Methods) prior to nuclease digestion.

digested with either S1 nuclease (16) or with RNAses A + T1 to detect the extent of hybrid formation. Approximately 66% of the nucleotides (Table 2) were found to be resistant to nuclease digestion. If samples were denatured and incubated at hybridization conditions prior to nuclease digestion their resistance increased to about 74%. Values obtained with the two types of nuclease digestion are in excellent agreement. The per-cent complementarity as estimated by nuclease resistance is in excellent agreement with acid-precipitable counts (Table 1) if the following correction is applied. If the background levels of acid-precipitable material observed in the absence of enzyme are subtracted from both the complete and minus template oligo(rU) primed reactions, then it is found that the addition of globin mRNA increases the production of RNA by 75%. That is, 75% of the total RNA present after one hour in the complete

reaction mixture is there as a result of the primer-template reaction, and 25% is the product of some non-specific Qβ replicase reaction. It is known that this enzyme can produce an endogenous "6s" product when incubated with various templates (4). The above calculation assumes that the reaction in the absence of globin mRNA shown in figure 1 is co-incident with the template-primer dependent reaction. Evidence supporting this assumption is presented below. The small increase in the percentage resistant to nuclease following a hybridization incubation indicates that most of the cRNA remains bound to the globin mRNA template following synthesis.

Electrophoretic analysis of cRNA. [^{32}P]- and [^{14}C]-labeled cRNA products were denatured and analysed on high resolution polyacrylamide-formamide slab gels, in parallel with highly purified [^{125}I]-labeled globin mRNA. Figure 2 is a composite of such data. Mobility in these gels is known to be linearly related to the logarithm of RNA chain length, as all RNAs are assumed to be completely denatured in the presence of 98% formamide (15). The transcription products of the Qβ replicase template-primer dependent reactions appear in discrete size classes (slots 2 and 3, fig. 2). This is also the case when cDNA copies are obtained from globin mRNA templates in the avian myeloblastosis virus reverse transcriptase reaction and may be a reflection of secondary structure along the globin mRNA chain (14). Slot 4 is a display of the RNA products obtained in the Qβ replicase reaction when globin mRNA is absent. It is seen, in comparison to slots 2 and 3 that the mRNA template programs the synthesis of the large number of discrete length products. While synthesis in the absence of template produces RNA of much shorter length (see below). The largest transcripts are not equal in length to the template, but are 570 NT long as measured by their mobility relative to the marker dyes and the known length of globin mRNA (650 NT) (15). The oligo(dT) primed reaction generates more

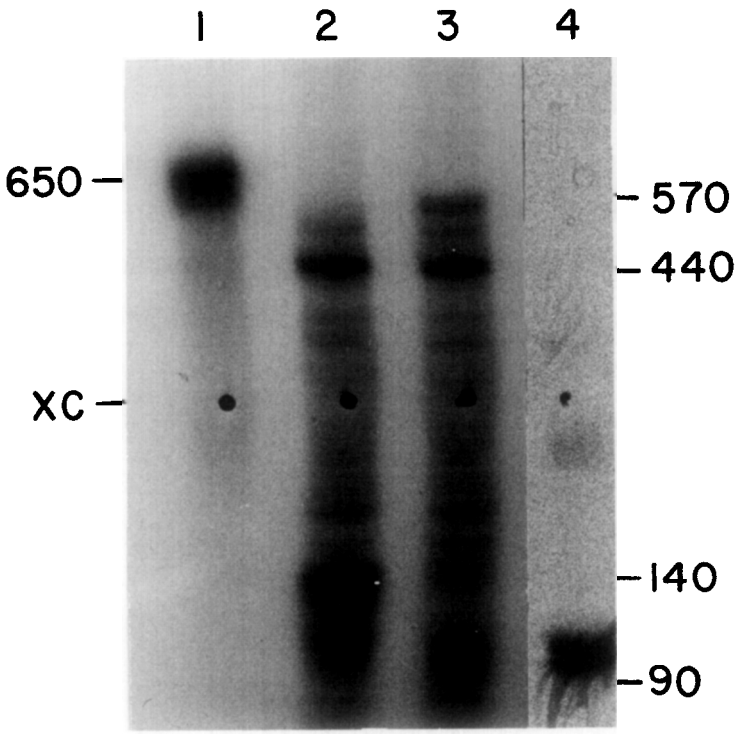

Figure 2. Autoradiogram of radioactively labeled cRNA electrophoresed on a 5% polyacrylamide-98% formamide gel. Slot 1: [^{125}I]-globin mRNA, Slots 2-3: [^{32}P]-cRNA products of the oligo(rU) and oligo(dT) primed reactions, respectively. Slot 4: [^{14}C]-labeled non-specific RNA products of an oligo(dT) containing reaction carried out in the absence of globin mRNA template. XC refers to the marker dye xylene cyanol.

material of maximum length than does the oligo(rU) one. The most prominent long band, in both reactions, is found at 440 NT, while the most abundant material in both template-primer dependent reactions is found in two zones between 90 and 140 NTs. The non-specific RNA synthesized in the absence of template (slot 4) is found in a zone of radioactivity of the same length as the prominent zones in slots 2 and 3. The range of lengths in these zones (i.e. 90-140 nucleotides) is consistent with RNA designated as "6s" from sucrose gradient analysis (4). The additional bands displayed in slots 2 and 3 are therefore presumed to be cRNA copies of globin mRNA of discrete but partial length.

Isolation of single-stranded labeled cRNA. The [^{32}P]-labeled true cRNA transcripts contain either oligo(rU) or oligo(dT) sequences at their 5'-termini, whereas the unlabeled template contains poly(A) at its 3' end. About 67%-74% of the reaction product is found to be a double-stranded hybrid by nuclease resistance criteria (Table 2). However, if the reaction product is denatured in formamide, heated, and diluted 20 fold prior to oligo(dT)-cellulose chromatography (see Methods), it is found that the S1 nuclease resistance of the labeled material drops to about 37% (Table 2). Under these conditions the S1 resistance of [^{125}I]-globin mRNA is about 30% (J.V., unpublished results), possibly reflecting the existence of specific secondary structure in the labeled mRNA. If analogous resistant regions are present in the cRNA, the lowered nuclease resistance represents a substantial decrease in the fraction of labeled cRNA in hybrid form. Therefore, oligo(dT)-cellulose affinity chromatography is a potentially useful method for preparing radioactive globin cRNA in pure form.

Conclusions. The data presented in this paper clearly demonstrate the ability of Qβ replicase to synthesize RNA complementary to globin mRNA in the presence of oligo(rU) or oligo(dT) primers. The reaction generates some nearly full length and many partial discrete cRNAs that remain in a hybrid form with the globin mRNA template unless specifically separated. The reaction also generates considerable non-specific RNA of length between 90-140 nucleotides. Efforts are in progress to completely characterize this reaction. For example, it is important to understand why the rather high background values are obtained. The Qβ replicase template-primer dependent reaction can potentially provide a simple method for obtaining highly radioactively-labeled cRNA for sequencing, and for use as specific sequence probes, in cases where it is not easy to obtain labeled mRNA.

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REFERENCES:

1. Kamen, R. I. (1970) Nature 228, 527-533.
2. Kondo, M., Gallerani, R. and WEissman, C. (1970) Nature 228, 525-527.
3. Haruna, I. and Spiegleman, S. (1965) Proc. Nat. Acad. Sci. U.S. 54, 579-587.
4. Weissman, C., Billeter, M. A., Goodman, H. M., Hindley, J. and Weber, H. (1973) Ann. Rev. Biochem. 42, 303-328.
5. Hori, K., Eoyang, L., Banerjee, A. K. and August, J. T. (1967) Proc. Nat. Acad. Sci. U.S. 47, 1790-1797.
6. Haruna, I. and Spiegleman, S. (1965) Proc. Nat. Acad. Sci. U.S. 54, 1189-1193.
7. Palmenberg, A. and Kaesberg, P. (1974) Proc. Nat. Acad. Sci. U.S. 71, 1371-1375.
8. Obinata, M., Nasser, D. S. and McCarthy, B. J. (1975) Biochem. Biophys. Res. Commun. 64, 640-647.
9. Feix, G. and Hake, H. (1975) Biochem. Biophys. Res. Commun. 65, 503-509.
10. Kamen, R. I. (1972) Biochim. Biophys. Acta 262, 88-100.
11. Blumenthal, T., Landers, T. A. and Weber, K. (1972) Proc. Nat. Acad. Sci. U.S. 69, 1313-1317.
12. Nienhuis, A. W., Falvey, A. K. and Anderson, W. F. (1974) Methods in Enzymology 30, K Moldave and L Grossman eds., 621-630.
13. Efstratiadis, A. and Kafatos, F. C. (1975) Methods in Molecular Biology ed. J. Last 8, Marcel Dekker: New York, in press.
14. Efstratiadis, A., Maniatis, T., Kafatos, F. C., Jeffrey, J. and Vournakis, J. N. (1975) Cell 4, 367-378.
15. Maniatis, T., Jeffrey, A., van de Sande, H. (1975) Biochem. 14, 3785-3794.
16. Britten, R. J., Graham, D. E. and Neufeld, B. R. (1974) In Methods in Enzymology 29 E. L. Grossman and K. Moldave, eds. (New York: Academic Press), 363-406.
17. Vogt, V. N. (1973) Eur. J. Biochem. 33, 192-200.