PRIMER DIRECTED INITIATION OF RNA SYNTHESIS CATALYSED BY QB REPLICASE

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Summary: It was found that, in addition to the well established primer independent initiation, RNA synthesis catalysed by Qß replicase can also be started by the presence of high concentrations of oligonucleotides complementary to the template, thus allowing the copying of RNAs otherwise not utilized as template by Qß replicase. This new type of initiation was tested with synthetic polyribonucleotides as templates.

Qß replicase displays high specificity with respect to the initiation of RNA synthesis. The initiation step is primer independent and is based on specific interactions of the enzyme with distinct parts of the template RNA. As a result, only a few RNAs are accepted as templates (1). In an attempt to reduce this limitation, we found that the in vitro RNA synthesis catalysed by Qß replicase can also be started with the help of suitable oligonucleotides which act as primers. This new type of initiation circumvents the highly specific normal initiation mechanism and allows the copying of RNAs otherwise not utilized as template. Besides its practical application for the in vitro copying of RNAs, the primer directed initiation studies may also be helpful for a further analysis of the function of the various proteins of Qß replicase (2).

Materials: Ribonucleoside triphosphates were bought from Böhringer. ^{3}H-labelled ribonucleoside triphosphates were products of Amersham (England). $(rU)_2$ and $(rU)_7$ were from Böhringer, $(rI)_5$ from Miles. $(rU)_{15-20}$ was isolated from a limited alkaline hydrolysis of poly U followed by alkaline phosphatase treatment, as described recently (3).
5'^{32}P-labelled $p(rU)_7$ and $p(rI)_5$, prepared with the help of polynucleotide kinase as described recently (3), were kindly supplied by Dr. H. Sano of this department. Poly A, poly I and poly C were purchased from Böhringer. The average molecular weight of the polymers was approximately 3×10^5 daltons. The Qß replicase preparation purified according to the procedure of Kamen (4) has been recently described (3).

Methods: Standard assay of Qß replicase reaction: The reaction mixtures contained 80 mM Tris-HCl (pH 6,9 at 30°C), 12 mM $MgCl_2$, 1 mM EDTA, 0,4 - 0,6 mM ribonucleoside triphosphates, 60 units/ml Qß replicase and templates and primers as specified in the individual experiment. After the various incubations described in the legends of the figures and tables samples were taken and analysed for acid precipitable radioactivity as described recently (3).

Molarities of nucleotides, oligo- and polynucleotides were determined spectrophotometrically with a Zeiss PMQ spectrophotometer using known molar extinction coefficients and neglecting hyperchromicity effects of the polymers.

Results: The basic properties of the primer dependent initiation of RNA synthesis were investigated with poly A - $(rU)_7$ as a template-primer combination. Other combinations were also tested and exhibited a similar behaviour. Fig. 1A shows that in the presence of 25 μM primer and at a template concentration of 0.22 μM one template equivalent has been copied after 90 minutes of incubation. Together with the finding that most of this product was of template size, as analysed by gel filtration chromatography under denaturating conditions (data not shown), this could mean that RNA synthesis starts at or close to the 3'end of the template and stops after one round of synthesis in this case. Further addition of enzyme or substrate after reaching the plateau did not stimulate more RNA synthesis. Fig. 1A also shows that no synthesis took place in the

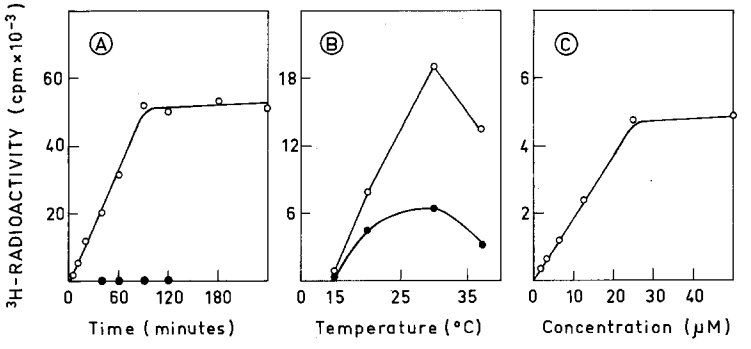

Figure 1

Effect of incubation time (A), incubation temperature (B) and concentration of oligo $(rU)_7$ on the copying of poly A.

(A) The 60 μl standard incubation mixture contained 25 μM oligo $(rU)_7$, 0.4 mM [³H]-UTP (7.5 x 10^4 cpm/nmole) and 0.22 μM poly A (o-o-o) or no poly A (•-•-•). After a incubation at 30°C for the times indicated in the figure 3 μl samples were taken and further analysed for acid precipitated radioactivity as described in methods. (B) 25 μl standard incubation mixtures containing 0.22 μM poly A, 25 μM oligo $(rU)_7$ and 0.4 mM [³H]-UTP (3 x 10^4 cpm/nmole) (o-o-o) or containing 0.3 μM poly C and 0.4 mM [³H]-GTP (1.5 x 10^5 cpm/nmole (•-•-•) were incubated at the temperatures indicated in the figure. 5 μl samples taken after 20,40 and 60 minutes were processed as in (A). The 40 minutes values are plotted. (C) 30 μl standard incubation mixtures containing 0.22 μM poly A, 0.4 mM [³H]-UTP (2 x 10^4 cpm/nmole) and oligo $(rU)_7$ at molarities as given in the figure were incubated at 30°.
5 μl samples taken after 10,20,30 and 60 minutes were processed as in (A). The 20 minutes values are plotted.

absence of poly A. The temperature optimum was around 30°C, and was sharper than in the case of a poly C dependent reaction as shown in figure 1B. The primer concentration is an important feature of the reaction since the reaction rate was greatly reduced even at concentrations which represent still surplus amounts of primer on a molar basis (Fig. 1C). It should be stressed that for any other template-primer combination the kinetics and concentration dependencies may be slightly different and should be analysed before further application. Replacement of Mg^{++} by 1 mM Mn^{++} reduced the enzyme activity to 10 per cent as opposed to the primer independent reactions with Qβ RNA (5) and poly C (6). The $(rU)_7$ dependent reaction worked over a wide range of UTP concentrations; 0,4 mM was used routinely. The length of the oligonucleotide primer is important as indicated in table 1. While $(rU)_7$ operates well at 30° (see figure 1B), $(rU)_2$ is almost inactive at 30°C. At 20°C $(rU)_2$ shows a better priming effect although at higher concentrations the overall activity is still very low. $(rU)_{15-20}$ was an ineffective primer at all concentrations tested. The behaviour of the various primers may reflect the necessity of short time hybrid formation between template and primer. Stable hybrid formation as in the case of $(rU)_{15-20}$ may be unfavourable in agreement with the finding that the RNA product of

TABLE 1

Oligo $(rU)_n$ dependent copying of poly A

No	Primer	^3H -UMP incorporated (nmoles/ml)	
		40 minutes	60 minutes
1	$(rU)_2$	1.5	3.2
2	$(rU)_2$	4.0	14.7
3	$(rU)_7$	128.5	165.7
4	$(rU)_{15-20}$	2.0	3.0

5 µl samples of standard incubation mixtures containing 0.22 µM poly A, 0.4 mM [^{3}H]-UTP (3 x 10^4 cpm/nmole) and 25 µM of the indicated oligonucleotides were taken after incubation at 30°C for the given periods of time and analysed as described in methods. Reaction No. 2 was incubated at 20°C. The oligo $(rU)_{15-20}$ of reaction No. 4 was present at a molarity of 10 µM.

normal Qß replicase reactions is not hydrogen bonded to the template RNA (7). In the case of poly C as template, the efficiency of the primer directed synthesis can be directly compared with the primer independent reaction. This is possible because GTP is indispensable for the initiation of the primer independent reaction while it can be replaced by ITP in the elongation steps. Thus, in the absence of GTP, no primer independent synthesis takes place (Reaction No. 1 of table 2). From reactions No. 2 and 5 it can be seen that the $(rI)_5$ initiated reaction is already after 20 minutes of incubation twice as efficient as the GTP started reaction. Table 2 also shows that the primer directed synthesis is not impaired by the use of a replicase sample previously treated with trypsin (Reaction No. 3). This trypsin treatment almost exclusively splits the GTP binding subunit III of replicase into two smaller peptides as revealed by SDS-polyacrylamid gel electrophoresis under denaturating conditions (data not shown). Such a trypsin treatment of replicase lowered however the primer independent activity of Qß replicase.

To see whether the primer is covalently linked to the product RNA 5' ^{32}P-labeled primers were used for the incubation with replicase. The reaction products were then analysed in a chromatographic system which separates oligonucleotides from polynucleotides. In the particular experiment described here

TABLE 2

Oligo $(rI)_5$ dependent copying of poly C

No	XTP	oligo $(rI)_5$	^3H- rXMP incorporated (nmoles/ml)	
			20 minutes	40 minutes
1	ITP	-	0.6	0.6
2	ITP	+	21.6	31.5
3	ITP	+	20.5	22.5
4	ITP	+*	0.5	0.5
5	GTP	-	10.0	12.0
6	GTP	+	13.1	13.9

6 µl aliquots of standard incubation mixtures containing 0.3 µM poly C, 0.6 mM [^3H]-GTP (1 x 10^5 cpm/nmole) or 0.6 mM [^3H]-ITP (2.5 x 10^4 cpm/nmole), and 25 µM oligo $(rI)_5$ were taken after a incubation at 30°C for 20, 40 and 60 minutes and processed as described in methods.
The replicase used for reaction No. 3 was incubated before the assay for 30 minutes at 20°C with RNAse free trypsin (30 µg/ml) in the presence of 20 mM Tris-HCl (pH 8.0). The trypsin was not removed for the replicase assay.

*The $(rI)_5$ of reaction No.4 was 3'phosphate terminated.

the replicase was trypsin treated as described above. Experiments with untreated replicase gave the same result.

Figure 2 shows that under the conditions of this experiment 0.57 p moles of $(rI)_5$ corresponding to 14% of the moles of poly C in the assay mixture were incorporated into the product as indicated by the radioactive peak at the origin of the chromatogram (part A). In the case of $(rU)_7$ and poly A the values were 0.37 p moles and 11% (part C). The control experiments performed without enzyme are depicted in part B and D. A further indication for the covalent linkage of the primer with the product is the complete inability of

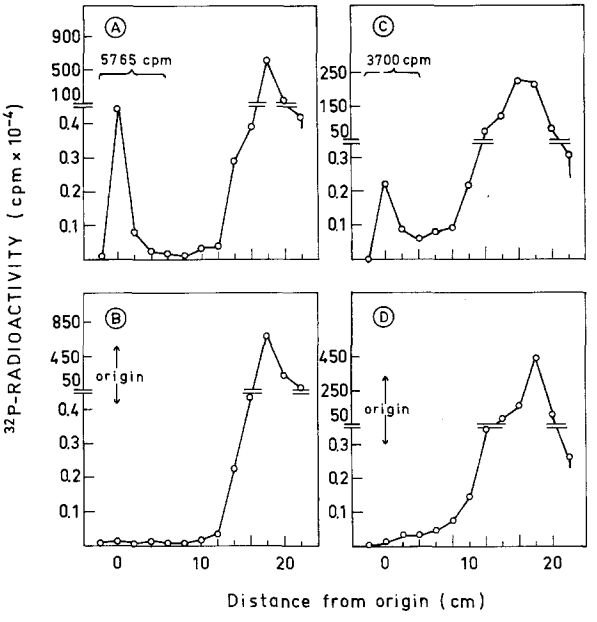

Figure 2

5' ^{32}P -labelled oligoribonucleotide primer incorporation into polyribonucleotide product:

30 µl standard incubation mixtures containing 0.13 µM poly C, 20 µM 5' ^{32}P-Oligo p $(rI)_5$ (1 x 10^7 cpm/nmole) and 0,6 mM ITP (part A and B of the figure) or containing 0.11 µM poly A, 20 µM 5' ^{32}P -oligo p $(rU)_7$ (1 x 10^7 cpm/nmole) and 0.6 mM UTP (part C and D) were incubated for 60 minutes at 30°C. The replicase used for the incubation mixtures of part A and C was trypsin treated as described in the legend to table 2. The incubations of part B and D were performed without replicase. After the incubation the solutions were brought to 40 mM EDTA and 7 M urea and chromatographed in ascending fashion on 20% DEAE cellulose thin layer glass plates at 55°C with homomix C according to Brownlee et al.(9). After drying 1 cm zones of the cellulose were removed sequentially from the glass plate, suspended in toluene scintillation mix and counted for radioactivity. These values are plotted in the figure.

3' phosphate terminated oligonucleotides to serve as primers (table 2, reaction No. 4).

Discussion: Because of the high specificity of the initiation mechanism, Qß replicase utilizes only a few RNA species as template for the in vitro RNA synthesis (1). The new type of primer directed initiation, described in this publication, will allow the copying of all RNAs for which suitable complementary oligoribonucleotides are available in appropriate amounts. As tested with synthetic polyribonucleotides as template almost all template molecules in the assay mixture are copied under the proper conditions. The reaction with trypsin treated Qß replicase showed that the primer dependent activity functions also without intact subunit III. This finding may stimulate further studies towards an understanding of the function of the subunits of replicase. So far no primer dependent RNA polymerases have been described except in the case of E.coli RNA polymerase which operates on a DNA template and shows primer dependent behaviour only at low substrate concentrations (8). It is further distinguished from Qß replicase in that it accepts dinucleotides as normally functioning primers (8).
Since high concentrations of oligonucleotides are needed for the primer dependent reactions of replicase this activity may not have an in vivo relevance. It will however be very useful for in vitro application, especially since there are no similar enzymes available that work on RNA templates.

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References:

1. Weissmann, C., Billeter, M., Goodman, H.M.,Hindley, J. and Weber, H. (1973) Ann. Rev. of Biochem. 43, 303-328

2. Landers, T.A.,Blumenthal, Th. und Weber, K. (1974) J. biol. Chem. 249, 5801-5808

3. Feix, G. and Sano, H. (1975) Europ. J. of Biochem.,submitted

4. Kamen, R. (1972) Biochim. Biophys. Acta 262, 88-100

5. Palmenberg, A. and Kaesberg, P. (1974)
Proc. Nat. Acad. Sci. USA 71, 1371-1375

6. Mitsunari,Y. and Hori, K. (1973)
J. Biochem. (T) 74, 263-271

7. Weissmann, C., Feix, G. and Slor, H. (1968)
Cold Spring Harbor Symp. Quant. Biol. 33, 83-100

8. Hoffman, D.J. and Niyogi, S.K. (1973)
Proc. Nat. Acad. Sci. USA 70, 574-578

9. Brownlee, C.G. and Sanger, F. (1968)
Eur. J. Biochem. 11, 395-399