POLYDEOXYRIBONUCLEOTIDES AS TEMPLATES FOR RNA SYNTHESIS CATALYSED BY Qβ REPLICASE

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1. Introduction

Qβ replicase, the replicating enzyme of RNA bacteriophage Qβ, accepts only a few polyribonucleotides as template for RNA synthesis in vitro [1]. So far, the molecular basis for this high template specificity is unknown even though the subunit structure of the enzyme, as well as the nucleotide sequence of the relevant parts of some template RNAs, have been analysed [2,3]. Therefore, it was of interest to study the influence of the sugar moity of the polynucleotide on the template specificity and to compare the template efficiency of polyribonucleotides with the corresponding polydeoxyribonucleotides. Since, however, the deoxysequences corresponding to the natural template RNAs are not available, we took advantage of the poly(rC) dependent Qβ replicase activity [4,5] by using poly(dC) as template for approaching the question. We found that poly(dC) is recognized as a template by Qβ replicase and can also be used as an initiator sequence for further RNA synthesis.

2. Materials and methods

Ribonucleoside triphosphates and poly(rC) were bought from Boehringer (Germany), ^{3}H-labelled ribonucleoside triphosphates from Amersham (England). Poly(dC) and poly(dA) were synthesized with terminal deoxynucleotidyl transferase (= addase) according to Kato et al. [6]. Poly(dA)$_{30}$–(dC)$_n$ was prepared correspondingly with (dA)$_{30}$ (synthesized with addase) as a primer and was separated from unused (dA)$_{30}$ by gel filtration. fd DNA fragments with a (dC)$_n$ sequence at their 3′end (fd-(dC)$_n$) were prepared by using fd DNA fragments (about 8s, obtained by limited DNAse digestion of intact fd DNA) as a primer for the addase catalysed addition of dCMP residues. The elongated fd fragments were purified from unused fd fragments by sedimentation in a sucrose gradient. Rabbit globin RNA (from Searle, England) with a (dC)$_{12-18}$ sequence at the 3′end was obtained by using the 3′terminal poly(rA) of globin mRNA as the primer for the *E. coli* DNA polymerase I catalysed copying of template (dG)$_{12-18}$ (dT)$_n$ (synthesized from dG$_{12-18}$ (from Collaborative Research, USA) as the primer by addase catalysed addition of dTMP units).

As analysed by sedimentation centrifugation, the elongated globin mRNA (globin RNA - [^{3}H]-dC)$_{12-18}$) remained intact under the conditions of the DNA polymerase reaction (incubation for 2 h at 15°C with 10 units/ml polymerase (Klenow form, product of Boehringer) 120 μg/ml globin RNA, 10 μg/ml dG$_{12-18}$ (dT)$_n$ and other reagents as described by R. Roychoudhury [7]). The synthesized globin RNA-(dC)$_{12-18}$ was used in the replicase assay without prior removal of remaining globin RNA. The Qβ replicase preparation was purified according to the procedure of Kamen [8]; it was free of RNAse and RNA polymerase. The standard Qβ replicase assay as well as the analysis of the acid precipitable radioactivity were performed as outlined earlier [9].

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

3. Results and discussion

The template activity of poly(dC) was investigated in standard incubation mixtures containing [^{3}H]GTP as the substrate by measuring the acid insoluble radioactivity. Incubations with poly(rC) as the template were performed in parallel. Fig.1 shows that compared with the poly(rC) reaction, the poly(dC) reaction exhibits a similar kinetic behaviour and a comparable concentration dependency. Incubations without template showed no incorporation (fig.1B). Under the conditions of the experiment the template efficiency of poly(dC) is approximately one third that of poly(rC). Replacement of Mg^{2+} by Mn^{2+} does not increase the template response of poly(dC) (data not shown). In order to verify that the poly(dC)

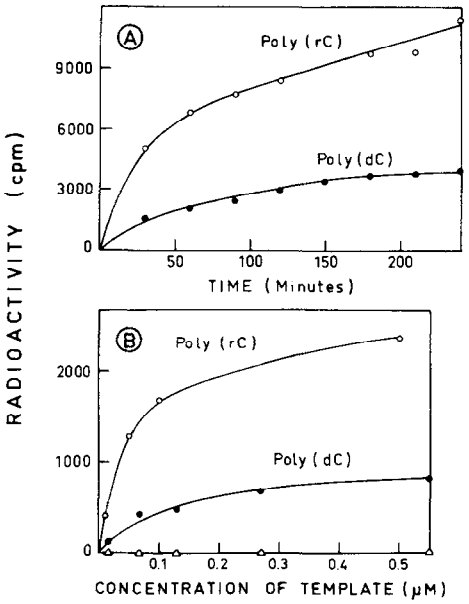

Fig.1. Effect of incubation time (A) and concentration of template (B) on [^{3}H]GMP incorporation. (A) Standard assay mixtures of 100 μl containing 0.8 mM [^{3}H]GTP (5 × 10^4 cpm/nmol) and 0.3 μM of poly(rC) (o-o-o) or poly(dC) (•-•-•) were incubated at 30°C. At the time indicated 5 μl samples were taken and analysed for acid-insoluble radioactivity as described in Materials and methods. (B) Standard assay mixtures of 30 μl containing 0.8 mM [^{3}H]GTP (1.5 × 10^4 cpm/nmol) and poly(rC) (o-o-o), poly(dC) (•-•-•) or no template (△-△-△) at concentrations as indicated were incubated at 30°C. 5 μl samples were taken after 0, 30, 60 and 90 min of incubation and processed as described in (A). The 60 min values are shown.

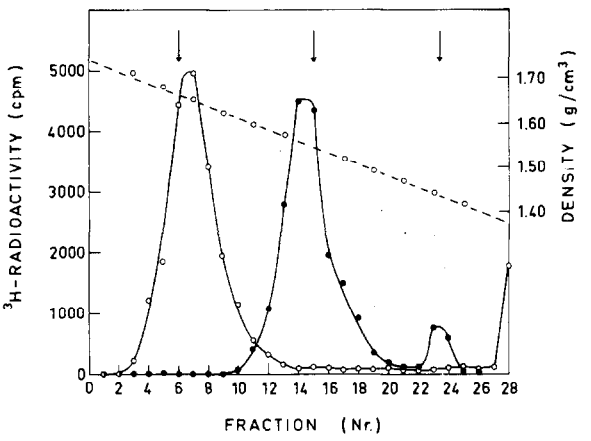

Fig.2. Cs_2SO_4 density gradient centrifugation of enzymatic products. 50 μl incubations as described in fig.1A were incubated for 3 h, then adjusted to 20 mM EDTA and 1% SDS and phenolized. An aliquot of these solutions was added to a mixture of 4.4 ml TE buffer (0.1 M Tris-HCl, (pH 7.6), 0.005 M EDTA) and 4 ml TE buffer saturated with Cs_2SO_4. After overlayering with oil the solutions were centrifuged in a TI 50 Beckman Rotor for 66 h at 44 000 rev/min at 15°C. One part of the gradient fractions was analysed for acid-insoluble radioactivity, the other part was taken for measuring the refractive index. (o-o-o) = poly(rC) as template, (•-•-•) = poly(dC) as template. The arrows indicate the positions of double-stranded RNA, DNA-RNA hybrid and double-stranded DNA, respectively, (taken from published data).

template indeed directs the synthesis of poly(rG), the buoyant density of the enzymatic product was analysed in a Cs_2SO_4 density gradient. Fig.2 shows that the ^{3}H-radioactivity of the incorporated GMP residues bands at a density of 1.55 which is characteristic for DNA–RNA hybrids, thus demonstrating conclusively that poly(dC) serves as a template for the synthesis of the complementary poly(rG). The results of a parallel Cs_2SO_4 gradient performed with the product of a replicase incubation with poly(rC) as the template, which leads to double stranded RNA, is included in fig.2. It may be concluded from these experiments that the main structural features, which lead to the recognition by Qβ replicase, are similar in poly(dC) and poly(rC). In an attempt to extend these template studies to other polydeoxynucleotides, we attached a deoxycytidine sequence to the 3′end of various polydeoxynucleotides hoping that the RNA synthesis would be initiated at this dC sequence in

Table 1
Incorporation of ^{3}H-nucleotide in response to various templates

Substrate	Templates 1	2	3	4	5	6
	poly(dA)	$(dA)_{30}-(dC)_n$	fd-DNA_{linear}	fd-$(dC)_n$	globin–RNA	globin–RNA-$(dC)_{12-18}$
	(counts/min)					
[a] GTP + UTP	–	4010	–	– –	–	–
GTP + [a] UTP	–	360	–	– –	–	–
[a] UTP	0	60	–	– –	–	–
[a] GTP + ATP / UTP + CTP	–	–	0	1120	–	–
GTP + ATP / [a] UTP + CTP	–	–	0	0	–	–
GTP + [a] ATP / UTP + [a] CTP	–	–	–	–	0	1520[a]

Standard assay mixtures of 30 µl containing 0.8 mM of the indicated ribonucleoside triphosphates and 50 µg/ml of the indicated templates were incubated at 30°C. The triphosphates marked with an asterisk were ^{3}H-labelled (1×10^5 cpm/nmol). 5 µl samples were taken after 0, 30, 60 and 90 min of incubation and analysed for acid-insoluble radioactivity. The 60 min values are listed.
[a] In the case of globin RNA-$(dC)_{12-18}$ as the template the incorporation continued with incubation time as shown by other experiments (e.g. 2900 cpm. after 3 h).

analogy to recent findings with polyribonucleotides [9].

The results of ^{3}H-nucleotide incorporation experiments in response to these polynucleotides are summarized in table 1. The respective radioactive nucleotides are marked by an asterisk.- It is demonstrated that the copying of poly(dA) by Qβ replicase can be achieved under standard conditions only if the RNA synthesis is started at the poly$(dC)_n$ tail attached to the 3′end (column 1 and 2). Similar results were obtained with $(dT)_n-(dC)_n$ (data not shown). In the case of a natural DNA, however, the RNA synthesis apparently does not proceed into the heteronucleotide region after starting at the 3′ terminal $(dC)_n$ sequence (column 3 and 4). The reason for this failure of Qβ replicase to copy natural DNA as opposed to certain deoxyhomopolynucleotides may lie in a still unknown property of the enzyme complex. If, however, the 3′ terminal deoxycytidine sequence is linked to an RNA rather than to a DNA, the poly(dC) initiated RNA synthesis is extended into the heteronucleotide region of the template (column 5 and 6). The further product analysis of this reaction with globin RNA–$(dC)_{12-18}$ as the template gave the same results as already described for the oligo$(rU)_7$-primer dependent copying of globin RNA by Qβ replicase [10]. This shows that indeed the heteronucleotide part of the RNA has been copied. Thus, the initiation of RNA synthesis at a $(dC)_n$ sequence located at the 3′end of an RNA may be of practical use for the copying of RNAs otherwise not accepted by Qβ replicase. However, the frequent concommittant 6s RNA synthesis occuring in Qβ replicase incubations, may interfere with the application of Qβ replicase in $(dC)_n$ initiated reactions [11].

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