Deletion Analysis of Qβ Replicase

PARTICIPATION OF THE CARBOXYL-TERMINAL REGION OF THE β-SUBUNIT PROTEIN IN TEMPLATE RECOGNITION

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We have analyzed one of the functional domains of Qβ replicase, an RNA-dependent RNA polymerase of RNA coliphage Qβ. Deletion mapping analysis of the carboxyl-terminal region of the β-subunit protein revealed that the terminal 18 amino acid residues (positions 571–588) are dispensable for the replicase reaction. Subsequent deletions up to the Ala-565 residue reduced the RNA polymerizing activity of the replicase in vitro but increased it in vitro. The mutant replicates with enhanced in vitro RNA polymerizing activity were found to have relaxed template specificity for ribosomal RNAs and cellular RNAs as well as Qβ RNA. Deletions beyond the Ile-564 residue abolished both the RNA polymerizing activity and the binding ability to midvariant (MDV)-polY(+) RNA, a derivative of a natural template for Qβ replicase, MDV-1 RNA. These results suggest that the carboxyl-terminal part of the β-subunit participates in RNA recognition of Qβ replicase.

With the development of modern gene technology, it became possible to analyze the relationships between the structures and functions of gene products. In the case of RNA coliphages, the complete nucleotide sequences of representative phage genomes were determined (1–4), and analysis of the structure of each gene also became possible. In attempts to clarify the functional domain of the polymerases, several polymerase genes have been extensively characterized.

An RNA-dependent RNA polymerase (RNA replicase) of RNA coliphage Qβ consists of four subunits. Three of them are host-derived proteins (ribosomal protein S1, and protein elongation factors, EF-Tu and EF-Ts) (5). Only the β-subunit, which is composed of 588 amino acid residues, is encoded by phage RNA. The central region of the β-subunit is well conserved in RNA coliphages, and this region is thought to contain common structural features for such as the assembly of the subunits and catalysis for RNA synthesis (6). A sequence motif, Gly-Asp-Aasp, which has been identified in RNA-dependent RNA polymerases of many viruses and is considered to be the active site for polymerization (7, 8), is located in the central part of the β-subunit protein (9). Recently, this motif sequence of the Qβ and polio virus 3Dpol proteins was found to take part in the binding of metal ions necessary for RNA polymerization (10, 11).

The Qβ replicase can specifically transcribe in vitro the Qβ RNA as well as the RNA from closely related phages (5). S1 and another host protein, HF-I (12), are required for the recognition of only Qβ viral plus-strand RNA as a template. In contrast, Qβ replicase lacking S1 and HF-I transcribes polY(+) RNA, MDV-1 RNA, and Qβ minus-strand RNA, as does the holoenzyme of Qβ replicase (5). RNA replicases from phages MS2, GA, and SP share the host subunits of Qβ replicase except for HF-I, but they have the different template specificity. Therefore, it is reasonable to think that some factor(s) determining the enzyme specificity must be present in the β-subunit.

The β-subunit proteins of MS2 and GA replicases are approximately 10% shorter than those of Qβ and SP replicases, with most of the extra amino acids being located in the carboxyl-terminal region (4). Furthermore, the carboxyl termini of the β-subunit are more divergent in sequence (Fig. 1). It is therefore likely that this region may contribute to template discrimination.

To clarify in detail the physiological role of the carboxyl-terminal part of the β-subunit in the Qβ replicase reaction, we constructed a series of plasmids harboring the β-subunit gene with deletions at its 3′-terminal end and examined the effects of the deletions on the enzyme functions. The carboxyl-terminal 18 amino acid residues were found to be dispensable for the replicase reaction, but a larger deletion made the enzyme defective.

EXPERIMENTAL PROCEDURES

Bacteria, Phage, and Plasmids—Escherichia coli NA (sup proF+) and BE110 (sup+ rel-I T2 supA22 lacA4 Hfr) were used as indicators. E. coli 594F ΔlacI (sup+ gal Stxl recA44/F° lacZI° lacI° proAB) was used as a recipient for transformation and for growing phages. Phage Qβus51 was described previously (13). Plasmid pRQ1, which contains the wild-type β-subunit gene of Qβ replicase, was used to construct plasmids carrying various sizes of the replicase gene lacking the 3′-terminal region. Plasmid pUC-MDV-LR carrying the segment corresponding to MDV-polY(+) RNA was described previously (10). MDV-polY(+) RNA is 244 nucleotides long and is a derivative of naturally occurring MDV-1(+)+ RNA (14), for which Qβ replicase exhibits strong template activity. Plasmid pQβs-32 (15) was provided by C. Weissmann.

Materials—Poly(rC) and DNase I were purchased from Pharmacia Biotech Inc. [γ-32P]ATP, [α-32P]dCTP, [α-32P]GTP, and [α-32P]UTP were obtained from Amersham Corp. Restriction endonucleases were purchased from TaKaRa Shuzo. AmpliTaq DNA polymerase was from Perkin-Elmer. The 16 and 23 S ribosomal RNAs of E. coli MRE600 were from Boehringer Mannheim.

Construction of Plasmids Carrying the β-Subunit Gene—Plasmids carrying the β-subunit gene, of which the 3′-terminal end was shortened to various extents, were constructed using the polymerase chain reaction method. Oligonucleotide RQ1-22 has a nucleotide sequence corresponding to positions 3328–3351 of the Qβ cDNA sequence including the cleavage site for the restriction enzyme, XhoI, and was used as a forward primer (Table I). The oligonucleotides listed in Table I were used as reverse primers, containing an artificial stop signal leading to premature termination of β-subunit protein synthesis and having the site for BglII for inserting the DNA fragment into the vector plasmid. Amplification of the DNA fragments was carried out according to the
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Fig. 1. Amino acid sequence similarity of the carboxyl-terminal region of the β-subunit protein among MS2, GA, Qβ, and SP replicase. Each amino acid is represented as a single letter. Numbers start from the amino-terminal end of the β-subunit protein. Asterisks indicate identical amino acids. Bars are used to represent probable insertion or deletion sites. Sequence data are taken from the Refs. 1–4.

Table I

| Oligonucleotide primers used for constructing deletions at the COOH-terminal end of the wild-type β-subunit protein |
|-------------------------------------------------|
| Oligonucleotide | Nucleotide sequence | Carboxy-terminal amino acid |
|-----------------|---------------------|-----------------------------|
| RQ1-2           | 5′-GTACACATTCCGAGCTGACGTCG-3′ | Leu-512 |
| RQ1-3           | 5′-CGGATCTAAGGATGCTAGATACG-3′ | Ser-534 |
| RQ1-4           | 5′-CGGATCTAAGAATGCGACCAGATG-3′ | Ser-567 |
| RQ1-5           | 5′-CGGATCTAATTTGCCGGTAGACACGT-3′ | Leu-569 |
| RQ1-6           | 5′-CGGATCTAATGCCAGTATGAGATACG-3′ | Ser-569 |
| RQ1-7           | 5′-CGGATCTAAACACGGCTACTGCAC-3′ | Tyr-570 |
| RQ1-8           | 5′-CGGATCTAACGGCTACTGCACGCG-3′ | Tyr-574 |
| RQ1-9           | 5′-CGGATCTAATACTGTATATCGAATTTG-3′ | Ser-581 |
| RQ1-10          | 5′-CGGATCTAATCGAATTTGCCGGTAG-3′ | Gln-582 |
| RQ1-11          | 5′-CGGATCTAAGACCTGCCTTATCTTCG-3′ | Gln-585 |

* Underlined sequences indicate the termination signals in the reverse primers.

* Numbers indicate the amino acid positions in the wild-type β-subunit protein, where the protein synthesis was terminated artificially at the mutant gene.

Amino acid numbers start from the amino-terminal end of the wild-type β-subunit protein. Asterisks indicate identical amino acids. Bars are used to represent probable insertion or deletion sites. Sequence data are taken from the Refs. 1–4.

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| RQ1-5           | 5′-CGGATCTAATTTGCCGGTAGACACGT-3′ | Leu-569 |
| RQ1-6           | 5′-CGGATCTAATGCCAGTATGAGATACG-3′ | Ser-569 |
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at 37 °C, the cells were spun down and resuspended in an equal volume of YT broth containing 2 mM isopropyl-1-thio-β-D-galactopyranoside, and then the incubation was continued for the indicated times. RNAs were extracted with phenol and precipitated with ethanol in the presence of 0.8% LiCl. The pellets were washed twice with 70% ethanol and then dissolved in distilled water. A 5-μg sample of the extracted RNA was denatured with glyoxal and dimethyl sulfoxide and then applied to a 10% agarose gel.

Electrophoresis, transfer to a nylon membrane (Biodyne A, Pall Ultrafine Corp.), and hybridization were performed as described previously (13). A 1.2-kilobase pair XhoI-BamHI fragment of ZpQβ-32 DNA, which lies within the maturation (A2) gene, was labeled using E. coli DNA polymerase I (Klenow fragment), random oligonucleotide primers (nonamers), and [α-32P]dCTP and then used as the hybridization probe to detect Qβ viral RNA.

Gel Retardation Assay—The 5′-end of MDV-poly(+) RNA was labeled as described previously (10). Ice-cold binding mixture (15 μl) comprised 50 mM Tris-HCl (pH 7.5), 0.1 mM dithioerythritol, 5% glycerol, 1.5 × 10−5 mol of 32P-end-labeled MDV-poly(+) RNA, and 1 μl of various cell lysate supernatants. After 20 min of incubation at 5 °C, samples (10 μl) were applied to a 5% polyacrylamide gel at 4 °C as described by Werner (18). The gel was then dried and subjected to autoradiography.

FIG. 2. Synthesis of the β-subunit proteins in the cells carrying the β-subunit gene with deletions. Lysates were prepared from E. coli 594/F+ lacP cells carrying pUC8 (lane b), pRQ1-DS534 (lane c), pRQ1-DS555 (lane d), pRQ1-DK558 (lane e), pRQ1-DD560 (lane f), pRQ1-DQ562 (lane g), pRQ1-DY563 (lane h), pRQ1-DI564 (lane i), pRQ1-DS565 (lane j), pRQ1-DC566 (lane k), pRQ1-DS567 (lane l), pRQ1-DA565 (lane m), pRQ1-DR568 (lane n), pRQ1-DV570 (lane o), pRQ1-DA572 (lane p), pRQ1-DV576 (lane q), pRQ1-DQ578 (lane r), and pRQ1-DL585 (lane s), or pRQ1 (lane t). A 5-μl sample of each cell lysate was applied to a 7% SDS-polyacrylamide gel. Dots indicate the position of the β-subunit protein. The Roman numerals next to lane a indicate the positions of the marker proteins: phosphorylase B (92.5 kDa) (I), bovine serum albumin (66.2 kDa) (II); ovalbumin (45.0 kDa) (III).

FIG. 3. Effects of amino acid deletions on Qβ replicase activity. A, in vivo complementation activity of the cells carrying the mutant plasmids. The percentage of activity was calculated on the basis of the phage titer for cells carrying pUC8. The frequency of phage adsorption was more than 80% for every clone. B, in vitro RNA polymerase activity. The relative RNA polymerase activity (ng/reaction) of cell lysates was determined on the basis of the amount of [32P]UMP incorporated in the reaction mixture containing 100 ng of purified Qβ replicase using Qβ RNA (darkened bars) or MDV-poly(+) RNA (open bars) as a template. Values are the means ± S.D. for two independent determinations; bars indicate S.D. Footnote 1, lysate of cells carrying pUC8. Footnote 2, lysate of cells carrying pRQ1.
ing phage genomes.

Also seen were the hybridization signals at the positions near to those of 16 and 23 S rRNAs (Fig. 4, lanes f and h–j). These signals may represent the premature transcripts of Qβ RNA because such small RNAs were observed in Qβ RNA-dependent RNA synthesis by wild-type or DS567 replicase (see Fig. 5C).

Qβ RNA Polymerase Activity—Using lysates from cells carrying various mutant plasmids described above, we examined Qβ RNA-dependent RNA polymerizing activity.

As shown in Fig. 3B, as long as the amino acid deletion was less than 18 residues, the polymerizing activity of lysates from the cells carrying the mutant plasmid was similar to that of the control cell lysate. However, when the deletion extended beyond the Val-570 residue of the β-subunit, despite the fact that the cells expressing the mutant replicases supported poor growth of the invading Qβsus51 phage (Fig. 3A), lysates of the cells carrying pRQ1-DR569, -DS568, -DS567, or -DC566 synthesized much more RNA than did lysate from cells harboring pRQ1. These results suggest that those deletions did not affect the polymerizing activity of the replicase. When 25 or more amino acids were deleted from the carboxyl terminus of the β-subunit protein, polymerizing activity was abolished. In addition, lysates of cells carrying pUC8 exhibited little RNA polymerizing activity.

To further analyze the characterization of the mutant replicase, we purified DS567 replicase and compared the Qβ RNA-dependent RNA synthesis by wild-type and DS567 replicases. As shown in Fig. 5A, when 16 ng of the template Qβ RNA was added to the reaction mixture, DS567 replicase synthesized RNA in a similar manner and amount to wild-type replicase. However, when 1 µg of the template RNA was incubated with DS567 replicase, incorporation of [32P]UTPs was about 2-fold of that using wild-type replicase. Fig. 5B shows the relationships between Qβ RNA-dependent synthesis and the molar ratio of Qβ RNA to replicase (Qβ RNA/repli- case). Since Qβ replicase binds to Qβ RNA at three sites (5), an excess amount of Qβ RNA causes binding of Qβ replicase to two or three Qβ RNA molecules, resulting in inhibition of Qβ RNA synthesis (20). Under our experimental conditions, Qβ RNA-dependent synthesis was found to be maximum at a molar ratio of about 0.3 or 0.5 for the wild-type or DS567 replicase reaction, respectively. When the ratio was higher than 0.5, DS567 replicase synthesized RNA twice as much as wild-type replicase did, which was consistent with the results in Fig. 4A. When the [32P]-labeled RNAs synthesized by wild-type or DS567 replicase at a molar ratio of 0.5 were compared by an agarose gel electrophoresis, the positions of Qβ RNA, 16 and 23 S ribosomal RNAs were determined as in Fig. 4.

**Fig. 4. Northern blot analysis of RNAs from cells infected with phage Qβsus51.** Phage Qβsus51 was introduced into E. coli 584/F lacF cells carrying pUC8 (lanes c and d), pRQ1-DA565 (e and f), or pRQ1-DS567 (g and h), or pRQ1 (i and j) and incubated for another 20 min (c, e, g, and i) or 40 min (d, f, h, and j). Lane b represents mock infection of cells carrying pUC8. Five-µg samples of RNAs were applied to the gel, except for in lane a, where 12 µg of Qb phage RNA was applied. 16 and 23 S RNAs of E. coli MRE600 were electrophoresed in a lane on the gel, transferred to a nylon membrane, and their positions were determined by cutting the lane of the membrane and staining it with methylene blue.

**Fig. 5. Effects of the ratio of Qβ RNA to replicase on the Qβ RNA-dependent RNA synthesis.** A, time course of RNA synthesis. The reaction mixture (50 µl) contained 0 µg (shaded symbols), 0.016 µg (open symbols), or 1 µg (closed symbols) of Qβ RNA and 500 ng of wild-type (circles) or DS567 (squares) replicase. Values are the means ± S.D. for two independent determinations; bars indicate S.D. B, relationships between the molar ratio (Qβ RNA/repli- case) and Qβ RNA synthesis. The reaction mixture (50 µl) contained 200 ng of wild-type (circles) or DS567 (squares) replicase and the indicated amounts of Qβ RNA. Incubation was performed at 37 °C for 10 min. 200 ng of the replicase was equivalent to 0.86 pmol of the holoenzyme, and 1 µg of Qβ RNA was equivalent to 0.70 pmol of the RNA. C, gel electrophoresis of the RNAs synthesized. The RNA synthesis was carried out at 37 °C for 10 min (lanes a and c) or 20 min (lanes b and d). Equal amounts of radioactive RNA (1,100 cpm) synthesized by DS567 (lanes a and b) or wild-type (lanes c and d) replicase at the molar ratio of 5 were denatured and applied to each lane of a 1% agarose gel. After electrophoresis, RNAs were transferred to a membrane (Biodyne A). The positions of Qβ RNA, 16 and 23 S ribosomal RNAs were determined as in Fig. 4.
trophorese, most of the 32P-labeled RNAs were found to be similar in size to Qβ viral RNA (Fig. 5C). These results indicate that the polymerization activity of DS567 replicase did not get damaged and suggest that DS567 replicase had an extra ability to bind to Qβ RNA.

**Template Specificity of the Mutant Replicases**—It was puzzling that lysates of cells carrying pRQ1-DC566, -DS567, -DS568, and -DR569 exhibited higher polymerizing activity in vitro with the Qβ RNA template but that these cells were unable to support effectively the proliferation of the invading phage, Qβssus51. Therefore, we examined the polymerizing activities of cell lysates using cellular RNAs as templates. As shown in Table II, lysates of cells carrying pRQ1-DC566, -DS567, -DS568, or -DR569 incorporated more [32P]UTPs compared to the control cell lysates carrying pRQ1 when ribosomal RNA or total cellular RNA was used as template. In contrast, lysates of cells carrying pRQ1-DA572 or -DV576, which exhibited lower Qβ RNA polymerase activity in vitro but supported progeny production in Qβssus51-infected cells, had lower polymerization activity than the control lysate in the cellular RNA-dependent system.

To further determine the cause of the difference between the in vitro and in vitro replicate reactions, we compared the template specificity of purified wild-type and DS567 replicases. Qβ replicase transcribes closely related phage RNAs but not other RNAs such as ribosomal RNAs and unrelated phage RNAs (5). As shown in Table III, when SP RNA, a closely related phage RNA to Qβ RNA, was also used as a template, DS567 replicase incorporated more [32P]UTPs than the wild-type replicase, as in the case of Qβ RNA template. DS567 replicase also exhibited much higher RNA polymerizing activity for 16 and 23 S ribosomal RNAs or total cellular RNA as template, confirming the results of Table II. These results indicate that DS567 replicase had relaxed specificity for not only Qβ RNA template but also cellular RNA templates.

**MDV-poly RNA and Poly(rG) Polymerase Activities**—Since Qβ replicase lacking S1 and HF-1 does not transcribe Qβ RNA but can use MDV-1 RNA and poly(rG) as template, we examined the effects of deletions on MDV-poly RNA and poly(rG) polymerase activities. The MDV-poly(+) RNA-dependent polymerizing activity of cell lysates changed in a similar way to Qβ RNA-dependent polymerizing activity as the amino acid deletion extended into the inner part of the β-subunit protein (Fig. 3B). When 25 residues were deleted, lysates of cells expressing mutant replicases with larger deletions no longer showed the polymerizing activity.

The poly(rC)-dependent polymerizing activity of cell lysates carrying pRQ1-DS568 decreased by 75% compared with the control cell lysates, as in the case of the MDV-poly(+) RNA template (Table IV). Furthermore, replacement of the carboxyl-terminal Ile residue of DS564 replicase with Ala or a chemically similar Leu residue reduced the poly(rG) and MDV-poly(+) RNA polymerizing activities to background levels. These results indicate that the sequence from Ile-564 to Ala-565 of the β-subunit protein is important for both the MDV-poly RNA and poly(rG) polymerase activities.

**MDV-poly(+) RNA Binding Activity**—Qβ replicase binds to the central region of MDV-1(+), MDV-2(+), and MDV-3(+) RNA, in which nucleotides 81–126 are identical with nucleotides 84–129 of Qβ minus-stranded RNA (21). To determine whether or not mutant replicases without MDV-poly RNA polymerizing activity can bind to a template, we examined a gel retardation assay using 32P-end-labeled MDV-poly(+) RNA. When the lysates of cells carrying pRQ1-DA565, pRQ1-DC566, or pRQ1 were incubated with [32P]MDV-poly(+) RNA and then electrophoresed on a polyacrylamide gel, the mobility of [32P]MDV-poly(+) RNA was decreased (Fig. 6, lanes c and g–i). In contrast, in the case of lysates of cells carrying pRQ1-DS567, which exhibited poor
polymerizing activity in cellular RNA-dependent synthesis (Table II). In contrast, lysates of cells expressing DA572 or DV576 replicase showed lower polymerizing activity than the control lysate in Qβ RNA or cellular RNA-dependent synthesis (Table II); however, these mutant proteins complemented the activity of phage Qβsus51 replicase (Fig. 3A). These results suggest that in cells infected with phage Qβsus51, a mutant replicase having a more relaxed template specificity than that of wild-type replicase engaged in transcribing cellular RNAs as well as Qβ RNA and accordingly failed to perform the task of polymerizing Qβ RNA, indicating that the accessibility of Qβ replicase to cellular RNAs may be critical for phage growth.

**MDV-poly(+) RNA Polymerase Activity**—DI564 replicase lacking the carboxyl-terminal 24 amino acid residues of the β-subunit protein showed only a little activity in the MDV-poly RNA polymerase assay (Fig. 3B). On removal of the terminal Ile residue from DI564 replicase, the resulting mutant, DY563, replicate lost the polymerizing activity (Table IV). Furthermore, DI564 replicase showed reduced binding ability to MDV-poly(+) RNA, and DY563 replicase abolished it completely (Fig. 6). These results indicate that the failure of MDV-poly(+) RNA-dependent RNA synthesis by DI564 replicase was due to poor binding of the replicase to the RNA. Therefore, the sequence from Ile-564 to Ala-565 of the β-subunit protein was essential for Qβ replicase to express MDV-poly RNA polymerase activity, particularly for its binding to MDV-poly(+) RNA. The finding that the insertion of a pentapeptide at the Ala-565 residue abolished the MDV-1 RNA polymerizing activity of Qβ replicase (6) agrees with our results.

Recently, Brown and Gold (22) have proposed a three-site model of Qβ replicase, according to which two RNA binding sites, site I on the S1 subunit and site II on EF-Tu, are responsible for RNA binding, and the polymerase-active site on the β-subunit is for RNA synthesis. Site I binds class I RNAs that possess single-stranded regions containing a high fraction of A and C nucleotides such as Qβ plus-strand RNA, and site II binds class II RNAs that have polypyrimidine tracts such as Qβ minus-strand RNA or MDV-1 RNA. According to their model, destruction of site II or removal of EF-Tu from Qβ replicase will result in the replicase without the MDV-1 RNA binding activity. Therefore, the data in Fig. 6 that show the importance of the Ile-564 and Ala-565 residues of the β-subunit protein in MDV-poly(+) RNA binding suggest that site II on EF-Tu interacts with these amino acid residues to form an active Qβ replicase molecule.

Our present results indicate that a change in the carboxy-terminal structure of the β-subunit protein of Qβ replicase could cause relaxation of the template specificity and that this part of the β-subunit is responsible for recognizing MDV-poly(+) RNA. The carboxyl-terminal region of the β-subunit protein is heterologous in RNA coliphages. Phage RNA replicase is thus assumed to have evolved its template specificity in part by altering the carboxy-terminal structure of the β-subunit and simultaneously by keeping the accessibility of the replicase to cellular RNAs at a low level.

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