DIRECT PARTICIPATION OF A HOST PROTEIN IN THE REPLICATION OF VIRAL RNA IN VITRO*

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An RNA-dependent RNA polymerase (replicase) induced by the RNA coliphage Q β has been purified¹ from infected cells and shown²⁻⁴ to be capable of synthesizing biologically competent viral nucleic acid. We have recently demonstrated⁵ that Q β -replicase can be separated into two components by repeated centrifugation in sucrose gradients. Their relative positions in the gradient indicate that one (designated "heavy") is approximately 130,000 and the other (designated "light") about 80,000 in molecular weight.

Neither of these components, adequately purified one from the other, can initiate extensive polynucleotide synthesis when challenged with intact Q β -RNA. Full activity requires a mixture of the two. However, the purified "heavy" fraction can synthesize poly G from guanosine 5'-triphosphate (GTP) if poly C is provided as a template—a reaction which may possibly reflect the fact that G is the initial base added^{6, 7} in the Q β -replicase reaction.

The size of the two replicase components raised a potential difficulty to the information available in the viral genome. The RNA of Q β has been shown^{8, 9} to be about 1×10^6 daltons and can, therefore, code for about 1×10^4 dalton equivalents of protein. Of this, 0.47×10^5 is accounted for by the viral coat protein and the attachment¹⁰ protein. This leaves only 0.53×10^5 equivalents to be shared between the two replicase units which sum to almost four times (2.1×10^5 daltons).

We do not know that sophistication of enzymatic function must necessarily be paralleled by an equivalent complexity in structure and variety of subunits. Thus, the apparent paradox is at least logically not insoluble. One could consider using combinations of small identical protein subunits to construct even highly sophisticated enzymes capable of catalyzing the complicated set of reactions characteristic of a replicase. Consequently, each replicase component could theoretically consist of identical subunits 4×10^4 in molecular weight, if all are the same, and about 2×10^4 if there are two different kinds. The information available in the viral genome would then be sufficient.

However, we earlier noted⁵ that another way to ease the apparent pressure for genetic information is to assume that one of the replicase components was a host-specified protein. Our previous experiments⁵ had shown that the "heavy" fraction was unique to infected cells. This suggested that the "heavy" protein was programmed in the viral RNA. However, neither genetic nor enzymological data had eliminated the possibility that the "light" component was a cellular protein. It seemed useful to entertain this possibility seriously and to explore its experimental consequences. It is the purpose of the present paper to present the resulting data.

The evidence to be described demonstrates the existence in uninfected cells of a

protein having all the properties of the "light" component of Q β - replicase. When combined with purified "heavy" component, an enzyme is reconstituted which can synthesize biologically functional Q β -RNA.

Materials and Methods.—(a) *Enzyme, substrates, and assays:* Purification of the Q β -replicase followed the minor modifications⁸ of the procedure of Haruna and Spiegelman.¹ The only change introduced in the present experiment is that infected cells were grown in L broth and the virus added at an OD₆₆₀ of 0.30. Infection was allowed to proceed as usual for 30 min at 37°C before the cultures were chilled with cracked ice and harvested.

The synthesis of α -P³²-riboside triphosphates, preparation of Q β -RNA, and assays for enzyme activity under standard conditions were as detailed previously.^{1, 5, 11}

(b) *Preparation and assay of "light" and "heavy" fractions:* Separation of the Q β -replicase into "light" and "heavy" fractions followed, with minor modifications, the procedure of Eikhom and Spiegelman.⁵ Enzyme from the *O*-(diethylaminoethyl)-step (DEAE-step) of the Q β -replicase purification¹ was rechromatographed on a DEAE-column. After concentration by precipitation with (NH₄)₂SO₄ and subsequent dialysis, the protein was layered onto a 5–20% sucrose gradient dissolved in 10% saturated (NH₄)₂SO₄, 0.02 M Tris pH 7.4, and 0.01 M MgCl₂. This was then centrifuged in rotor SB-260 of the International ultracentrifuge for 42 hr at 35,000 rpm and at 2°C.

The heavy component can be located either by its poly G-polymerase activity in the presence of poly C, or by its response to Q β -RNA in the presence of added "light." It should be noted that the poly C-dependent poly G-polymerization reaction cannot be employed to identify the Q β -replicase component until the DNA-dependent RNA polymerase (transcriptase) has been removed. The latter enzyme can also carry out this reaction.⁵ However, the transcriptase is readily separated from both Q β -replicase components by centrifugation in sucrose gradients. The "light" component can be located and assayed by its response to Q β -RNA in the presence of added "heavy." Once a component is located in the gradient, the relevant fractions are pooled, the protein concentrated by (NH₄)₂SO₄ precipitation, and after dialysis, rerun on a sucrose gradient in a Spinco SW39 at 5°C for 42 hr at 39,000 rpm. In some cases, a third run in the SW39 was added.

In experiments which required examination for the synthesis of infectious material, the CsCl-banding step of Pace and Spiegelman³ was included to decrease phage contamination prior to separation of the components on sucrose gradients. Samples for infectivity assays of the synthesized RNA were diluted into 0.003 M ethylenediaminetetraacetate (EDTA) and assayed with protoplasts as detailed previously.³

(c) *Preparation of "light" fraction from uninfected cells:* Uninfected cells (*E. coli* strains Q-13 and B) were grown up under conditions identical to those employed for infected cells, including the extra 30 min corresponding to the infection period. They were then chilled with ice at the same OD₆₆₀, harvested, and stored similarly at –14°C. The frozen plates were then subjected to precisely the same breakage and subsequent fraction procedure employed to isolate Q β -replicase through the stage of DEAE-chromatography. After concentration by (NH₄)₂SO₄ precipitation and subsequent dialysis, protein was subjected to centrifugation in sucrose gradients as detailed in section (b) above. Evidence for the presence of "light" activity was sought by testing each fraction with Q β -RNA in the presence of excess "heavy" purified from infected cells.

(d) *Electrophoresis in acrylamide gels:* Preswollen crosslinked 2.4% polyacrylamide were prepared as previously described.^{9, 12} Electrophoresis was carried out at room temperature for 90 min at 10 ma for gels 7 cm in length and 0.9 cm in diameter. Methods of preparing reaction samples and application to gels are as detailed earlier.¹³

Results.—(a) *Isolation of a "light" component from uninfected Q-13:* Uninfected cells of *E. coli* Q-13 were fractionated in a manner identical to that employed for the preparation of Q β -replicase.¹ The resulting protein from the DEAE-column was then centrifuged on a sucrose gradient by the procedure of

Eikhom and Spiegelman⁵ to separate the "heavy" and "light" components of Q β -replicase. The resulting samples were then assayed for "light" activity by their ability to stimulate purified "heavy" component to respond to Q β -RNA. Activity was detected in the same region of the gradient normally occupied by the "light" component of Q β -replicase. Active fractions were pooled, the proteins concentrated, and rerun on sucrose gradients as described in *Methods* (c). This second run is necessary to remove residual nucleases; for the same reason, the centrifugation is prolonged to 42 hours to achieve a better separation from most ribonucleases which tend to remain in the upper half of the tube. Figure 1 shows the results of assaying such a second gradient for "light" activity. The results leave little doubt that a protein component exists in noninfected cells which fractionates like the "light" component and can replace it in stimulating the "heavy" component of Q β -replicase to full activity.

In experiments of this nature it is of course necessary to ensure that the cells employed are in fact *not* infected with Q β -virus. This is by no means a trivial requirement nor easily satisfied in a laboratory engaged in growing large quanti-

FIG. 1.—Location in a sucrose gradient of "light" component activity from uninfected cells. Aliquots were assayed for "light" activity in the presence of added Q β -RNA and excess "heavy" component purified from infected cells. Reactions were carried out under standard¹ conditions for 20 min at 35°C. Values are corrected for the activity (720 cpm) of unsupplemented "heavy" component. The protein layered on the sucrose gradient (5–20%) was obtained from uninfected *E. coli* Q-13 cells fractionated according to the Haruna-Spiegelman¹ procedure for Q β -replicase and subjected to one prior sucrose-gradient purification—the centrifugation was carried out in a Spinco SW39 for 42 hr at 4°C and 39,000 rpm.

ties of RNA coliphages. These phages rapidly become ubiquitous and highly infectious environmental contaminants. In all experiments reported, non-infected cells were rigorously examined for the presence of Q β , and no batches were employed that showed detectable evidence of their presence.

To make doubly certain on this issue, one can employ F[−] strains (i.e., *E. coli* B) which cannot be infected by the RNA phages because these cells lack the F⁺ pili used as the specific route of infection. When this was done, we indeed found F[−] cells also contained a protein which behaved in all respects identically to the "light" component of Q α -replicase. Some of the experiments to be described below were performed with "light" fractions derived from extracts of *E. coli* B.

(b) *Some properties of the "light" fraction:* We now summarize experiments which demonstrate that the active component in the "light" fraction, derived

TABLE 1. *Nondialyzability of the "light" component.*

Source	Light Fraction	Treatment	Incorporation
Infected Q-13		None	5131
		Dialyzed vs. SB-AS	4937
		Dialyzed vs. SB	4724
Noninfected Q-13		None	4236
		Dialyzed vs. SB-AS	4180
		Dialyzed vs. SB	3894

"Light" component was prepared from infected and noninfected *E. coli* Q-13 as described in *Methods* (b, c). Portions (0.1 ml) were dialyzed for 24 hr against 1 liter of either SB (0.01 *M* Tris pH 7.4; 0.01 *M* MgCl₂) or SB-AS (SB + (NH₄)₂SO₄ to 10% saturation). In each case, 10 λ of "heavy" component (*Methods*, b) was challenged with 10 λ of the indicated light component. The reactions (125 λ) were run for 20 min at 35°C under standard conditions¹ using α -P³²-GTP at 1×10^6 cpm/0.2 μ M. The numbers represent counts per minute incorporated corrected for the activity (702 cpm) of unsupplemented "heavy" component.

TABLE 2. *Heat inactivation of "light" component.*

"Light" component source	Treatment	Incorporation (cpm)
Infected Q-13	None	3900
Noninfected B	None	2650
Infected Q-13	Heated—3 min	500
	Heated—5 min	350
Noninfected B	Heated—3 min	350
	Heated—5 min	300

The indicated light fractions were subjected to 100°C for 3 or 5 min, then rapidly cooled. All other details as in legend of Table 1. Again, the numbers represent counts per minute incorporated corrected for unsupplemented "heavy" component activity (600 cpm).

TABLE 3. *Sensitivity to trypsin of "light" component activity.*

Mixture	"Light" component incubated with	Cpm
A	Trypsin	233
B	Trypsin + trypsin inhibitor	3609
C	No additions	3805

The following reactions were carried out with three 100- λ aliquots of "light" component purified from *E. coli* B: (A) incubation with 2 μ g trypsin for 30 min at 35°C after which 10 μ g of trypsin inhibitor was added and allowed to incubate for 15 min at room temperature. (B) Incubation with a preincubated mixture of 2 μ g trypsin plus 10 μ g of trypsin inhibitor for 30 min at 35°C followed by a further 15 min at room temperature. (C) Control incubated with no additions for 30 min at 35°C and 15 min at room temperature.

In each case 10 λ of "heavy" was employed in the assay for light activity, 14 λ each of mixtures A and B, and 10 λ of the control mixture were employed. All other details are as in the legend of Table 1. Assays were carried out for 20 min at 35°C with α -P³²-GTP at 0.4×10^6 cpm per 0.2 μ M in a volume of 125 λ . Figures represent counts per minute incorporated corrected for unsupplementary "heavy" component activity (701 cpm).

from either infected or noninfected cells, possesses properties expected of a protein molecule. Table 1 shows that a 24-hour dialysis of "light" preparation against 10,000 volumes of buffer leads to no significant loss of activity. Exposure to 100°C for three minutes (Table 2) results in an 87 per cent loss of activity for the "light" component from infected Q-13 and an 88 per cent decrease for the preparation derived from uninfected cells (*E. coli* B).

The sensitivity of the "light" activity to trypsin was examined and the results are summarized in Table 3. After incubation with trypsin for 30 minutes at 35°C, the light fraction loses virtually all (94%) of its activity. If trypsin inhibitor is included during the exposure to trypsin, the activity remains intact.

The experiments summarized lead to the conclusion that the activity derived from uninfected cells is nondialyzable and sensitive to both heat and trypsin. Further, this protein can be isolated from uninfected cells by a procedure which yields a similar component from cells infected with the Q β -virus.

(c) *Nature of the product:* We now undertake to examine the nature of the product produced by combining the "heavy" component with "light" component obtained from uninfected cells. Figure 2 depicts an electrophoretic analysis on

FIG. 2.—Electrophoretic separation on acrylamide gels of RNA synthesized by "heavy" component supplemented with "light" component from uninfected cells. A 125- λ reaction containing 0.5 μ g Q β -RNA, 10 λ of "heavy" component and 10 λ of uninfected "light" was run for 20 min under standard¹ conditions at 35°C. Sodium dodecyl sulfate (SDS) was added to 0.2% and the sample applied to a 2.4% acrylamide gel which was run for 1.5 hr as described in *Methods* (c). The gels were then frozen, sliced, and counted as described previously.¹²

FIG. 3.—Synthesis of infectious RNA by a combination of infected "heavy" and uninfected "light" components. A standard¹ 1-ml reaction was set up using 1.5 μ g of Q β -RNA, α -P³²-UTP at 5×10^6 cpm/0.2 μ M; 30 μ g of "heavy" component separated from Q β -replicase carried through the CsCl and sucrose-banding steps² and 100 μ g of "light" component isolated from *E. coli* B. A similar control reaction was set up in which the "light" component was omitted. At the times indicated, 0.1-ml aliquots were removed, SDS added to 0.1%, pronase to 0.05%, and EDTA to 0.01 M. Samples were then diluted to 3.0 ml from which 0.2 ml was removed for infectivity assays³ and the remainder for assay of acid-precipitable incorporation.

acrylamide gels of the material synthesized in a 20-minute reaction by such a combination. One observes the FS¹⁴ and HS¹⁵ structures containing double-stranded RNA which precede¹⁶⁻¹⁸ by several minutes the appearance of Q β -RNA in the typical Q β -replicase reaction. In addition, an excellent peak of mature Q β -RNA is found. In general, when adequate amounts of "light" component are added, no matter what their source, the profiles obtained are indistinguishable from those seen with the usual Q β -replicase preparations.

A more rigorous, and necessary, criterion to be satisfied is "infectivity" of the product produced with "light" component from uninfected cells. The outcome

of such experiments is exemplified in Figure 3 in which a "light" fraction derived from *E. coli* B was used. It is evident that no new infectious RNA is produced *without* added "light" component. However, addition of *E. coli* B "light" component results in the synthesis of biologically active RNA as characteristically seen with normal Q β -replicase.

Discussion.—The discovery that one of the protein components of replicase is host-specified makes it easier to accommodate the viral-programmed proteins within the information provided by the viral genome. This finding is also more consistent with the demonstration¹⁹ of three complementation groups in the coliphage R-17. Since two are assigned to structural proteins (coat and attachment protein), only one is available for replicase.

Hori *et al.*²⁰ briefly noted finding a stimulatory factor for Q β -replicase which is resistant to heat (100° for 30 min) and does not appear to be a protein. Further, this factor (kindly supplied by Dr. J. T. August) does not stimulate our "heavy" component, nor does it have significant effects on our complete Q β -replicase reactions. The August-factor is clearly different from our "light" protein component, and its composition and relation to the Q β -replicase system remains to be delineated.

Rigorous proof that the "heavy" component of Q β -replicase is coded for by the viral genome will have to await the exhibition of a Q β mutant possessing an altered "heavy" protein. For the moment, we can accept this as the most likely possibility, supported by the observation⁵ that the "heavy" component is unique to cells infected with Q β . Lodish and Zinder²¹ have provided ingenious genetic experiments suggesting that conversion of the parental RNA into a double-stranded structure is carried out by a viral-specified enzyme and that this is the first enzyme to function. Bishop, Pace, and Spiegelman have recently shown⁷ that the first reaction to occur in the *in vitro* synthesis of Q β -RNA is a 5' to 3' polymerization of the negative complementary strand. This is then followed by a reversal of the chemical polarity leading to a novel 3' to 5' synthesis of new plus strands.

The genetic²¹ and enzymological evidence^{5, 7} would be most simply and attractively explained if the "heavy" component could carry out the 5' to 3' synthesis of the complement. The "light" protein could then be assigned to mediate, or participate in the second step, the 3' to 5' polymerization. Unfortunately, at present, the situation seems more complex. Thus far, initiation of extensive normal synthesis with Q β -RNA as a template has required the *simultaneous presence* of both "light" and "heavy" proteins. Further, the "light" protein has, to date, exhibited no detectable polynucleotide-synthesizing ability in the presence of either single- or double-stranded RNA. However, negative outcomes in such instances are not logically decisive.

The fact that we can obtain "light" protein from uninfected cells has simplified the task of its purification. Similarly, the finding that active enzyme can be reconstituted from its separable components has led to the development of a more efficient and effective purification of the "heavy" protein. These technical advantages are now making available highly purified components, permitting the design and performance of experiments that were not previously possible.

We were led to search for the "light" component of Q β -replicase in uninfected cells by the paucity of the information available in the viral genome. Finding this protein relieves one problem but clearly generates another and one with intriguing implications. To the best of our knowledge, cells uninfected with RNA viruses do not carry out RNA-dependent RNA syntheses. What, then, is the *cellular* function of a protein which can participate with a viral protein in the replication of RNA? The most obvious candidates for possible clues are the known DNA- and RNA-polymerizing reactions.

Summary.—We have previously⁵ shown that Q β -replicase is dissociable into "heavy" and "light" protein components which can be reconstituted into active enzymes. The present experiments show that a protein having all the properties of the "light" component can be isolated from *uninfected* cells, including strains (e.g., *E. coli* B) which are not hosts of the Q β -virus. "Light" protein from uninfected cells when combined with purified "heavy" protein yields an enzyme capable of producing biologically competent infectious Q β -RNA. Aside from its general interest to RNA virus replication, this finding generates another intriguing question, i.e., the *cellular* function of the "light" protein.

Note added in proof: Protamine is employed in the preparation of "light" component from both infected and noninfected cells. The possibility that protamine can activate the "heavy" component was examined over a wide range of concentrations with negative results.

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