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Bacteriophage fd Gene II-Protein

I. PURIFICATION, INVOLVEMENT IN RF REPLICATION, AND THE EXPRESSION OF GENE II*

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Gene II-protein of bacteriophage fd was purified from phage-infected *Escherichia coli* cells more than 5000-fold to near-homogeneity. The purification was performed on the basis of the ability of the protein to complement extracts of uninfected *E. coli* cells for DNA replication on fd RFI as template. The product of gene II-protein-mediated DNA synthesis on fd RFI was a full length viral strand. Gene II-protein also activated fd RFI for DNA synthesis with *E. coli* DNA polymerase I. Under native conditions and on sodium dodecyl sulfate gels the size of the protein was 46,000 daltons, which is in agreement with the molecular weight derived from the nucleotide sequence of bacteriophage gene II. Extracts from *E. coli* cells infected with phage fd mutants in gene V contained a high level of gene II-protein and the protein was lacking in cells infected with phage fd amber mutants in gene II. Comparison of the amounts of gene II-protein and fd RFI in fd-infected cells indicate a gene dose effect for the synthesis of gene II-protein. Gene II-protein was found to be a soluble protein, but it had a tendency to stick to membranous cell structures, when cells were grown at elevated temperature. Earlier *in vivo* experiments assigned an endonuclease function for gene II-protein. An accompanying paper (Meyer, T. F., and Geider, K. (1979) *J. Biol. Chem.* 254, 12642–12646) shows this activity to be specific for supercoiled phage fd RFI.

Infection of male *Escherichia coli* cells with filamentous bacteriophage (fd, f1, M13) results in the conversion of the single-stranded phage genome into the double-stranded replicative form. This step of DNA synthesis only depends on host proteins. The prominent components required for this stage were recently isolated from cell extracts (2). The next step, replication of the phage RF[1] to progeny RF relies on the action of a phage protein coded by gene II (3). This protein is also required during fd single strand synthesis. The isolated protein was found to participate in fd RF replication in a system which uses purified proteins (4).

When cells infected with filamentous phages were grown at 42°C, an increase of gene II-protein in membrane fractions of the cells could be demonstrated (5). Gene II-protein was also detectable when RF of filamentous phages was used as template in a protein-synthesizing system and it was absent when RF was used from amber mutants in gene II (6, 7). In both cases a protein band assigned to gene II-protein had the same molecular weight as the native protein, described in this paper.

Several types of regulation for the synthesis of gene II-protein have been proposed. We will show that the amount of the enzyme in infected cells correlates to the concentration of RFI in phage-infected cells.

MATERIALS AND METHODS

E. coli Strains and Bacteriophage fd Mutants

Strain H 560 is F^+, *pol* A1 *end* A1, tsx^r, str^r; H 570 is *pol* A1, *end* A1, *rns* A100, tsx^r, str^r; H 402 is strain H 570 *F'lac*; HF 4704 is *uvr* A, *thy*, phx^s; 1101 is F^+, *sup* E, *end* A1; Hfr C6 is *end* A1, *met*, sup^-, and Hfr C7 is Hfr C6 UVR. Phage fd *am* 51 is mutated in gene V, fd *am* 11 in gene II, and phage M13 *am* 8 in gene VIII. These phage mutants and strains H 560, H 570, HF 4704, 1101, Hfr C6, and Hfr C7 were from the collection of Dr. H. Hoffmann-Berling. Phage fd *ts* 11 is a temperature-sensitive revertant of fd *am* 11. Phage fd 11 was kindly provided by K. Neugebauer and Dr. H. Schaller. It is a wild type phage with an *Eco* RI site at the nucleotide position 5727.

Chemicals

Sources were as follows: Labeled nucleotides and [^{3}H]leucine from New England Nuclear; unlabeled nucleotides from Boehringer Mannheim; Bio-Rex 70 and hydroxylapatite (Bio-Gel HTP) from Bio-Rad; Ultrogel from LKB; the other chemicals from Merck.

Proteins and DNA

E. coli DNA-binding protein I was isolated as described (8). *E. coli* DNA polymerase I was eluted from a DNA cellulose column together with DNA-binding protein II (8). The eluate was further purified on DEAE-cellulose, phosphocellulose, and Ultrogel AcA 44 according to the method of Jovin *et al.* (9). Lysozyme and marker proteins were from Boehringer Mannheim and bovine serum albumin from Pentex. *In vitro* labeled proteins were prepared according to the method of Bolton and Hunter (10). Bovine serum albumin was treated with *N*-succinimidyl-3-(4-hydroxy,5-[^{125}I]iodophenyl propionate) and ovalbumin with *N*-succinimidyl[2,3-^{3}H]propionate (Amersham).

The preparation of phage RFI is outlined in the miniprint section, the sources of other DNA species were described elsewhere (1).

Assays for Gene II-Protein

Assay A: Complementation for RF Replication—For a 20-μl assay 0.2 μl of extract from uninfected cells (see miniprint section) was added to 0.1 μg of fd RFI, 0.5 mM ATP, 50 μM each of dATP, dCTP, dGTP, and 25 μM of [α-^{32}P]- or [^{3}H]dTTP (100 to 400 cpm/pmol of nucleotide), 5 mM $MgCl_2$, 20% sorbitol, 20 mM Tris·Cl (pH 7.5), 0.2 mM EDTA, 5 mM β-mercaptoethanol, and 0.6 μg of *E. coli* DNA-binding Protein I. Aliquots (2 μl) of fractions containing gene II protein were added and the mixture was incubated for 20 min at 30°C. DNA synthesis was measured as acid-insoluble radioactivity in a scintillation counter.

Assay B: Nick Translation with E. coli DNA Polymerase I—Alternatively, DNA synthesis with DNA polymerase I was measured

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[1] The abbreviations used are: RF, double-stranded circular DNA of replicative form; RFI, covalently closed, supercoiled RF; RFII, RF with a discontinuity in one strand; RFIV, covalently closed, relaxed RF; ssDNA, single-stranded DNA; moi, multiplicity of infection; BrdUTP, 5-bromodeoxyuridine triphosphate; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

after activation of fd RFI by gene II-protein. Extract, DNA-binding protein I and ATP were omitted from the assay described above and DNA polymerase I (0.2 μg) and a sample containing gene II-protein were added. Incubation was for 15 min at 37°C. Assays A and B were used in the purification of gene II-protein.

Assay C: Relaxation of Phage fd RFI (1)—A direct measurement of gene II-protein activity is the relaxation of phage fd RFI. This assay is therefore used to define the activity of the enzyme. One unit is the conversion of 1 pmol of fd RFI (4 μg) to Form II and Form IV in 15 min at 30°C. Details will be described in the accompanying paper (1).

Preparations of Membrane Fractions

Hfr C7 cells were grown at 37°C and UV-irradiated to suppress host protein synthesis (5). Then the cells were infected with phage fd and labeled with [^{3}H]leucine for 10 min at 37°C and further grown for 45 min at 42°C. Fractions from total membrane and fast sedimenting membranous material were prepared from spheroplasts of these cells according to the method of Osborn (11).

Other Procedures

Protein determination was carried out using the dye adsorption method of Glick (12). Double-stranded DNA cellulose was prepared according to the method of Litman (13).

Slab-gel electrophoresis. DNA was run on a 1% agarose gel in 40 mM Tris/acetate (pH 8.1), 1 mM EDTA at 3 V/cm. The gel was stained in a solution of 1 μg of ethidium bromide/ml of buffer and visualized in UV light. Proteins were run on polyacrylamide-SDS-gels (14). ^{3}H-labeled proteins were detected by fluorography of the dried gels (15).

RESULTS

Purification of Phage fd Gene II-Protein—An almost homogeneous preparation of gene II-protein was achieved in a procedure outlined in the supplementary material.[2] The isolation became possible by the use of phage fd mutants in gene V, showing an overproduction of gene II-protein and by the ability of the enzyme to complement extracts of uninfected cells to synthesize DNA on fd RF (Assay A). This system is similar to a test developed for *cis* A protein of phage φX 174 (16). Moreover, the unstable attachment of gene II-protein to the cleaved fd RF (1) allows DNA synthesis with DNA polymerase I at the nick (Assay B). This assay is less salt-sensitive than the complementation of cell extracts fd RF synthesis.

The purification steps included ammonium sulfate precipitation, a DEAE-cellulose flow through, chromatography on Bio-Rex 70, DEAE-cellulose, and dsDNA-cellulose, gel filtration and hydroxylapatite chromatography. The effluants of the columns with specific stimulation of DNA synthesis on fd RFI were further purified. The assays of the Bio-Rex column were in some cases disturbed by *E. coli* DNA binding protein II, which does not only inhibit DNA polymerases (8) and but also directly the activity of gene II-protein.[3] In this case gene II-protein activity was recovered after the following DEAE-cellulose column. About 200 μg of the enzyme were isolated from 1.7 kg of phage fd amber V infected cells with a yield of approximately 10% of the activity found in the ammonium sulfate precipitation of the cell extracts.

Purity of the Gene II-Protein Preparation—As demonstrated on SDS-gels (Fig. 1), gene II-protein Fraction VII was more than 90% pure. Contaminating endonucleases were not present, because gene II-protein activated only fd RFI as a substrate for *E. coli* DNA polymerase I and other DNA species were not affected by the addition of gene II-protein (Table I). Nucleases producing a 3′-phosphate end could also not be observed as the gene II-protein preparation did not cleave φX RFI (1).

Very low exonucleolytic degradation, which can be caused by a 5′-phosphatase, by exonuclease III, exonuclease VII, or the 3′ → 5′ exonuclease of DNA polymerases was detected in a transfection assay (see supplement). Contaminations by DNA ligase and unspecific topoisomerases (like ω protein) were excluded (1).

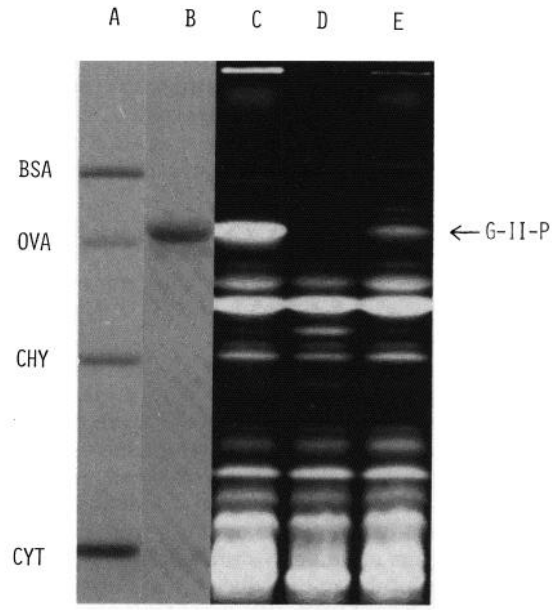

FIG. 1. **SDS-gel electrophoresis of gene II-protein.** Marker proteins were run in *Lane A*: *BSA*, bovine serum albumin (M_r = 68,000); *OVA*, ovalbumin (M_r = 44,000); *CHY*, chymotrypsinogen (M_r = 23,000); *CYT*, cytochrome *c* (M_r = 13,000). Gene II-protein (G-II-P) (10 μg, protein of Fraction VII) is shown in *Lane B*. *Lanes C*, *D*, and *E* show [^{3}H]leucine-labeled protein from fast sedimenting membrane fractions of phage-infected cells (see "Materials and Methods"). *Lane C*, fd *am* 51 (mutant in gene V) infected; *Lane D*: fd *am* 11 (mutant in gene II) infected; *Lane E*: fd wild type infected. The cells were grown at 42°C after infection. The polyacrylamide concentration in the gel was 12.5%.

TABLE I

Effect of gene II-protein on DNA synthesis with various DNA species

The assays were performed as described in Assays A and B for gene II-protein. DNA was applied at a concentration of 0.5 to 1 nmol of nucleotides/assay. The addition of 0.05 unit of gene II-protein (Fraction VI) is indicated in the column headings. All DNA species could be stimulated at least 10-fold in DNA synthesis by addition of 0.05 ng of DNase I/assay. RFIV was made from RFI with calf thymus nicking-closing enzyme (topoisomerase).

Gene II-protein	Extract system		DNA polymerase I	
	−	+	−	+
Template DNA	*pmol nucleotides incorporated*			
fd ssDNA	1[a]	1.5[a]	5	8
fd RFI	3	36	7	78
fd RFIV	9	12	10	16
φX RFI	4	4	11	14
ColE1-form I	7	9	11	14
T 7	16	16	11	14
λ	12	14	6	8
PM 2	5	5	5	6
SV40	12	10	12	13
None	1	1	2	2

[a] In the presence of 20 μg of rifampicin/ml to prevent initiation of ssDNA → RF conversion (2).

[2] Portions of this paper (including Figs. 1 and 2 and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document 79M-201, cite author(s), and include a check or money order for $1.80 per set of photocopies.

[3] T. F. Meyer and K. Geider, unpublished results.

Physical Properties—Protein of Fraction VII was analyzed by filtration on an Ultrogel AcA54 column. Gene II-protein activity eluted slightly faster than ovalbumin (M_r = 44,000) (Fig. 2 of supplement). In a glycerol gradient it was found between hemoglobin and cytochrome *c* at an S value of 3.7 (data not shown). These data suggest a globular shape for gene II-protein.

On SDS-gels, gene II-protein migrated at a slightly slower rate than ovalbumin corresponding to a molecular weight of 46,000 (Fig. 1). The coincidence of the molecular weight under native and denaturing conditions indicates that gene II-protein is a monomer in solution.

Identification of Gene II-Protein—When cells were infected with phage fd mutants in gene II (*am* 11), no gene II-protein activity could be measured even when the mutant-infected cells were treated with chloramphenicol (Table II). Fast sedimenting membrane fractions from cells infected with phage fd *am* 11 did not contain the 46,000-dalton protein, which was visible on SDS-gels in material from cells infected with wild type phage or amber V mutants (Fig. 1, Lanes D, C, E). This protein was also not produced in a protein-synthesizing system when phage fd RF with an amber mutation in gene II was used as template.[4]

Gene II-protein from Fraction VII co-migrates with gene II-protein from fast sedimenting membrane fractions (Fig. 1) and the observed molecular weight for the protein also agrees with data calculated from the nucleotide sequence of gene II (17). A partial amino acid sequence of labeled gene II-protein confirmed these findings.[5] In this case, labeled amino acids were incorporated in protein of phage fd-infected cells and gene II-protein recovered from membrane fractions (see Fig. 1). The positions of a specific amino acid in the protein were determined by an Edman sequenator.

DNA Replication with Gene II-Protein in Extracts—Among various DNA species checked for DNA replication in extracts of uninfected *pol* A mutant cells gene II-protein only stimulated the synthesis on fd RFI as template (Table I). No stimulation was observed for fd RFIV and fd ssDNA. Other supercoiled or linear DNAs were also unaffected for DNA synthesis in the presence of gene II-protein.

DNA replication on fd RFI in soluble cell extracts was asymmetric and only the viral strand was newly formed. After incorporation of BrdUTP the products of DNA synthesis were found in a cesium chloride gradient at a density which was expected for RF containing a heavy viral strand of full length and a light parental complementary strand (Fig. 2) (18). Thus, gene II-protein complements extracts from uninfected cells not only specifically in respect of the template fd RFI, but also initiates synthesis of the viral strand as it was previously shown for its *in vivo* function (3).

Expression of Gene II in Phage-infected Cells—A large increase of gene II-protein activity was found in cells infected with phage mutants in gene V compared to wild type-infected cells. In subsequent experiments on a possible regulation of gene II-expression a correlation of the gene II-protein level with the content of fd RFI in the phage-infected cells was observed (Table II).

In order to measure gene II-protein activity in cell extracts DNA was synthesized on fd RFI in extracts from cells infected with wild type phages and gene II-mutants. The level of DNA synthesis was as low as for extracts from uninfected cells. Increased synthesis was observed in the case of chloramphenicol treatment of wild type-infected cells. For the concentration used this drug interferes primarily with the synthesis of gene V-protein. The highest level of gene II-protein activity was found in cells infected with phage mutants in gene V.

In the previous experiments, gene II-protein activity was inferred from specific DNA synthesis in extracts. The protein can also be determined directly by gel electrophoresis of membrane fractions from phage-infected cells (Fig. 3). Gene II-protein and gene VIII-protein, which also accumulates in

[4] E. Fuchs, P. Hirth, T. F. Meyer, and K. Geider, unpublished results.

[5] T. F. Meyer, K. Beyreuther, and K. Geider, manuscript in preparation.

TABLE II

Gene II-protein and RFI in phage fd-infected cells

DNA Synthesis: Extracts (strain H 560) were prepared as described under "Materials and Methods." Assays were with 1 μl of Fraction II of infected cells as indicated in the table instead of extract from uninfected cells. Template was 100 ng of RFI. Gene II-protein: [^{3}H]leucine-labeled gene II-protein from membrane fractions (strain Hfr C7) was isolated as described under "Materials and Methods." The fluorographic band was compared with ^{3}H-standards. Phage fd RFI: The yield is from 6 liters of culture (strain Hfr C6) processed as described under "Materials and Methods."

E. coli cells infected with	DNA synthesis in cell extracts with		Gene II-protein from membrane fractions	Phage fd RFI recovered
	fd RFI	φX RFI		
	pmol nucleotides incorporated		*cpm*	*μg*
Uninfected	1.5	2.6		
fd wild type	2.1	1.9	2,000	190
fd wt + CAM[a]	4.0	2.1	ND	730
fd *am* 11	2.3	1.8	<200	<10
fd *am* 11 + CAM	1.7	2.2	ND	ND
fd *am* 51	21	6[b]	24,000	1,100

[a] CAM, chloramphenicol (30 μg/ml medium); ND, not determined.

[b] This value is probably higher than background due to the presence of fd RFI in Fraction II.

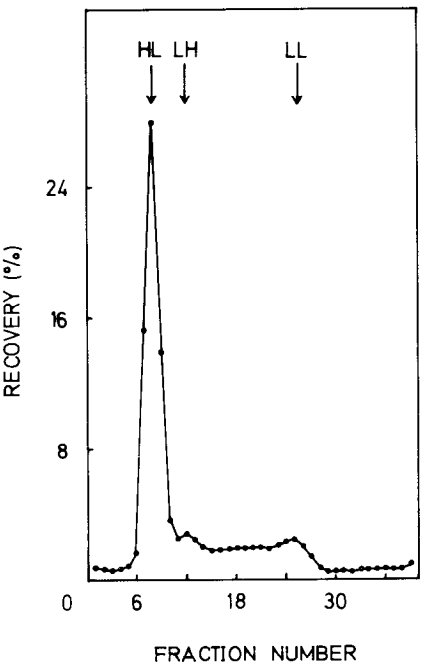

FIG. 2. **Equilibrium sedimentation analysis of bromouracil-labeled fd RF in neutral CsCl.** DNA was synthesized with [α-^{32}P]dATP (2000 cpm/pmol), 50 μM BrdUTP instead of dTTP and fd RFI as described in Assay A for gene II-protein ("Materials and Methods"). Gene II-protein was Fraction V. Internal references was fd RF with a bromouracil-substituted complementary strand and a light viral strand (*LH*) derived from ssDNA RF conversion with BrdUTP instead of dTTP (2). Light fd RF (*LL*) was used as external reference. Centrifugation was in a Beckman 50 Ti fixed angle rotor for 15 hours at 40,000 rpm and 20°C using the two layer technique (2).

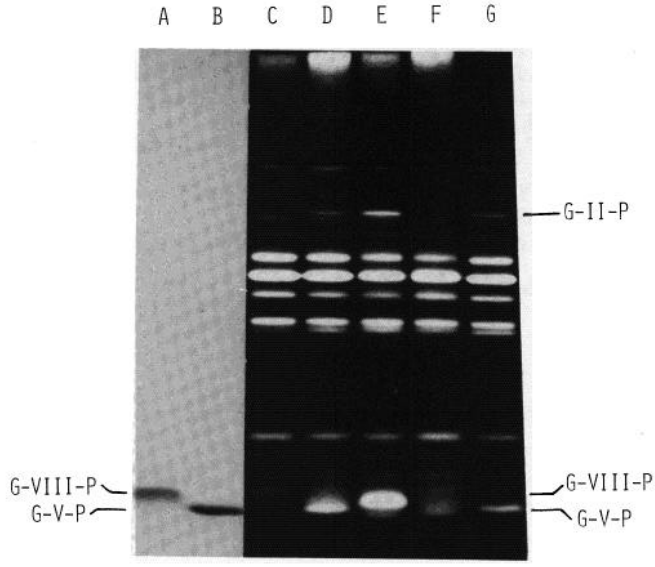

FIG. 3. **Expression of viral genes in fd mutant-infected cells demonstrated by SDS-gel electrophoresis of "total membrane fraction."** The preparation of the total membrane fractions from *E. coli* Hfr C7 is described under "Materials and Methods." *Lane C*, uninfected cells; *Lane D*, fd wild type infected; *Lane E*, fd *am* 51 infected, *Lane F*, fd *ts* 11 infected; *Lane G*, M13 *am* 8 infected. The gel contained a linear gradient from 12% to 20% acrylamide. The bands were made visible by fluorography. The migration of phage fd coat protein (*G-VIII-P*) as a dimer and fd gene V-protein (*G-V-P*) on this gel is shown in *Lanes A* and *B*, respectively. Gene II-protein (*G-II-P*) was identified by its migration similar to ovalbumin (Fig. 1).

membrane fractions, were strongly reduced in cells infected with fd *ts* 11 (gene II) at nonpermissive conditions (Fig. 3, Lane F). The wild type level of gene II-protein and gene V-protein (Fig. 3, Lane D) was not altered after infection with amber VIII-mutants (Fig. 3, Lane G). Only mutants in gene V showed high expression of gene II- and gene VIII-protein (Fig. 3, Lane E). The cells were shifted to 42° 10 min after infection, which strongly diminishes phage production. For wild type phages, only a small amount of gene VIII-protein should therefore get removed from the cells packaged with extruding phages.

Isolation of fd RFI from infected cells showed an increase of the RF from fd gene II-mutants via wild type-infected cells to fd amber V-mutants (Table II). Gene V-protein was diminished in this row, whereas gene II-protein and gene VIII-protein were increased (Fig. 3). We interprete these results as the influence of gene V-protein on fd RF accumulation. The amount of phage RFI determines then the expression of the phage genes. This view is supported by the finding that the lack of gene V-protein enhances both gene II-protein and gene VIII-protein.

Localization of Gene II-Protein in Infected Cells—Gene II-protein was readily recovered from the soluble proteins of phage-infected cells. Little gene II-protein was found in membrane fractions from cells grown at 37°C and little gene II-protein activity could be measured in extracts from cells grown at 42°C (data not shown). Therefore, we assume that gene II-protein is a soluble cytoplasmic protein which has a tendency to attach to the membrane. The enzyme is quickly inactivated at high temperature when measured in the endonuclease assay (1). This denaturation may also occur in the cell at 42°C which may result in the membrane association of most of gene II-protein.

DISCUSSION

This paper reports the isolation of biologically active bacteriophage fd gene II-protein, which was shown *in vivo* (3) and *in vitro* to be involved in the replication of double-stranded phage fd DNA. The enzyme was purified from *E. coli* cells infected with an fd amber mutant in gene V, which was found to be an overproducer for gene II-protein.

The isolated protein of 46,000 daltons is the phage gene II-product by following criteria: (i) The protein is not detectable on SDS-gels nor in a DNA synthesis assay from cells which were infected with fd mutants in gene II (Table II). (ii) The protein has the same molecular weight as gene II-protein resolved from membrane fractions or a protein-synthesizing system and agrees with the molecular weight calculated from the nucleotide sequence of gene II (17). (iii) The purified protein selectively cleaves supercoiled phage fd RF (1). (iv) Gene II-protein specifically stimulates DNA synthesis on phage fd RFI and not on phage ϕX RFI or other supercoiled or linear DNA when used for complementation of extracts from uninfected cells or DNA polymerase I (Table I). It exclusively promotes the replication of the viral strand.

The role of gene II-protein on phage fd RF replication (4, 19) is similar to the effect of *cis* A protein (20) in a system for phage ϕX 174 RF replication (16, 21).

Gene II-protein seems to be a cytoplasmic protein but it attaches to "membranous" cell structures at elevated temperature. The yield of the purified protein can be extrapolated to at least 200 molecules of soluble gene II-protein/cell infected with phage fd *am* V. This amount of gene II-protein is roughly equivalent to the cellular pool of RFI molecules.

Gene V-protein, a phage-coded DNA-binding protein, prevents the conversion of replicated viral strands to double-stranded DNA (2, 22). Subsequently, its absence in gene V mutant-infected cells increases the production of replicative form DNA. The higher yield of isolated fd RFI for amber V mutants relative to wild type corresponds approximately to the increase of gene II-protein and also of gene VIII-protein indicating a gene-dose effect for those proteins.

Other types of regulation have been proposed in the literature. According to a suggestion of Lin and Pratt (5), gene II-protein interferes with the expression of its own gene. This possibility appears less likely with the finding that a thermosensitive mutant in gene II does not overproduce the mutated gene II-protein under non-permission conditions. As another control mechanism gene V-protein was thought to repress directly the production of gene II-protein.[6, 7] Our results indicate that, besides the promotor strength, the cellular concentration of RFI is also responsible for the degree of viral gene expression.

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Additional references are found on p. 12641.

Supplemental Material

TABLE I. Purification of bacteriophage fd gene II-protein

Fractionation	Total protein	Complementation assay		Nick translation		Relaxation assay	
		Total activity (DNA synthesis)	Specific activity	Total activity (DNA synthesis)	Specific activity	Total activity	Specific activity
	mg	nmol	nmol/mg	nmol	nmol/mg	units	units/mg
I. High speed supernatant	110,000	-	-	-	-	-	-
II. Ammonium sulfate precipitate	19,200	7,300	0.15	3,800	0.2	12,000	0.6
III. DEAE-Cellulose pass-through, Bio-Rex 70 chromatography	280	3,100	11	1,240	4.5	3,900	14
IV. DEAE-Cellulose chromatography	48	2,800	58	1,130	24	3,950	82
V. dsDNA-Cellulose chromatography	9.9	1,300	131	950	96	2,800	280
VI. Ultrogel AcA44 filtration	0.8	(390)*	(500)*	830	1,160	2,400	3,000
VII. Hydroxyl-apatite chromatography	0.2	(140)*	(700)*	500	2,500	1,600	7,900

* Replication in extracts is diminished for higher purity of gene II-protein.

SUPPLEMENTAL MATERIAL TO:

BACTERIOPHAGE fd GENE II-PROTEIN. I. PURIFICATION, INVOLVEMENT IN RF REPLICATION AND THE EXPRESSION OF GENE II

by

T.F. Meyer and K. Geider

Purification of Gene II-Protein from Phage fd amber V infected *E.coli* Cells

E.coli H402 cells were grown at 37° in 140 l medium containing 15 g yeast extract, 15 g $Na_2HPO_4 \cdot 2H_2O$, 1.5 g KH_2PO_4, 5 mg vitamin B_1, 10 ml glycerol (87%) per l. At a density of A_{590} = 0.8 (5 x 10^8 cells per ml) the cells were infected with phage fd am 51 (moi = 50) and further aerated at 30° for 30 min. The culture was then chilled with ice and the cells were harvested by centrifugation. The cell paste was suspended in half its volume of 10% sucrose, 50 mM Tris·HCl (pH 7.5), frozen in liquid nitrogen and stored at -30°.

I. Extract: After thawing 250 g of the frozen cell preparation the suspension was diluted with 200 ml 10% sucrose, 50 mM Tris·HCl (pH 7.5), and adjusted to 20 mM spermidine, 5 mM β-mercaptoethanol, 1 mM EDTA, 0.1 M NaCl. Then 200 mg egg white lysozyme in 25 ml of the same buffer were added, and the cells were left on ice for 45 min. The incompletely lysed cells were slowly poured into liquid nitrogen and thawed in a water bath at 10°. The viscous lysate was centrifuged in a Beckman rotor 45 Ti for 1 h at 44,000 rpm and 0°.

II. Ammonium sulfate precipitation: The supernatant (Fraction I) was adjusted with Buffer A with 100 mM maleic acid to 500 ml. Protein was precipitated with 150 g $(NH_4)_2SO_4$, which was added within 10 min and stirred for 20 min at 0°. The precipitate was collected by centrifugation in a Sorvall GSA rotor at 13,000 rpm and -5° for 60 min. The pellet was dissolved by adding Buffer A with 100 mM maleic acid to a volume of 120 ml and the protein precipitated with 30 g $(NH_4)_2SO_4$. After keeping for 20 min in ice the suspension was centrifuged in a Sorvall SS 34 rotor at 20,000 rpm and -5° for 15 min and the supernatant discarded. The pellet could be stored frozen at -80° for several weeks (Fraction II).

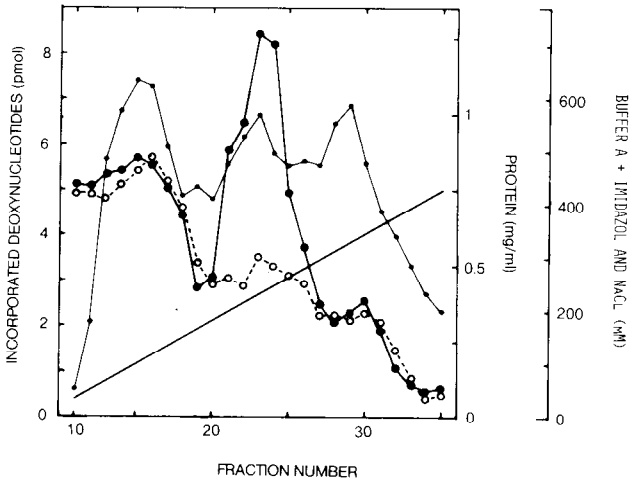

Fig. 1. Chromatography of Fraction II on a Bio-Rex 70 column. Gene II-protein activity was measured by DNA synthesis with *E.coli* DNA polymerase I according to Methods (●—●). Unspecific stimulation was determined with øX RFI (○---○) and the same method. Fractions preferentially active for fd RFI were pooled. The buffer strength (——) was measured by conductivity. Protein assays (•——•) were carried out by the procedure of Glick (2). Fractions were 5 ml.

III. Bio-Rex 70 chromatography: Fraction II was dissolved in Buffer A with 10 mM maleic acid and diluted with this buffer until the conductivity corresponded to the conductivity of Buffer A with 100 mM maleic acid. The solution was quickly pumped through a DEAE cellulose column (Whatman DE 52; 150 ml; 5 cm diameter, equilibrated in Buffer A with 100 mM maleic acid). The flow through was diluted during elution with an equal volume of Buffer A using a two channel pump and loaded on a Bio-Rex 70 column (40 ml; 2.6 cm diameter, equilibrated in Buffer A with 50 mM maleic acid). The DEAE cellulose column was washed with 200 ml Buffer A with 100 mM maleic acid, the Bio-Rex column uncoupled and washed with 120 ml Buffer A with 50 mM maleic acid. The bound protein was eluted from the Bio-Rex column in a 200 ml linear gradient of Buffer A with 50 mM maleic acid to Buffer A with 500 mM imidazol and 500 mM NaCl. Active fractions of the second peak (approximately the conductivity of Buffer A with 250 mM imidazol and 250 mM NaCl) in the assay with fd RF (Fig.1) were precipitated with $(NH_4)_2SO_4$ (0.35 g per ml), the protein was spun down and frozen (-80°) (Fraction III).

IV. DEAE cellulose chromatography: Ten preparations of Fraction III were pooled and the pellets were dissolved in 50 ml Buffer A with 25 mM imidazol and 25 mM NaCl, mixed with 50 ml DEAE cellulose (packed in Buffer A with 10 mM imidazol and 10 mM NaCl) and diluted with Buffer A with 10 mM imidazol and 10 mM NaCl until the suspension reached the conductivity of Buffer A with 25 mM imidazol and 25 mM NaCl. The suspension was applied on top of a DEAE cellulose column (100 ml; 2.6 cm diameter) in Buffer A with 25 mM imidazol and 25 mM NaCl. The column was washed with 500 ml Buffer A with 25 mM imidazol and 25 mM NaCl. The column was then developed with a 400 ml linear gradient of Buffer A with 25 mM imidazol and 25 mM NaCl to 250 mM imidazol and 250 mM NaCl. Gene II-protein eluted at the conductivity of Buffer A with 75 mM imidazol and 75 mM NaCl. Active fractions were pooled (Fraction IV).

V. DNA cellulose chromatography: Fraction IV was immediately loaded on a double strand DNA cellulose column (30 ml, 1.6 cm diameter) in Buffer A with 70 mM imidazol and 70 mM NaCl. After washing with 100 ml of the same buffer, the bound protein was eluted with a linear gradient of 200 ml Buffer A with 70 mM imidazol and 70 mM NaCl to 400 mM imidazol and 400 mM NaCl. Gene II-protein appeared at the conductivity corresponding to Buffer A with 220 mM imidazol and 220 mM NaCl. The peak fractions were precipitated with 0.5 g $(NH_4)_2SO_4$ per ml (Fraction V).

VI. Gel filtration: The pellet was dissolved in 2 ml Buffer B with 400 mM KCl, dialysed for 1 h against the same buffer and applied to an Ultrogel AcA 44 column (90 ml; 1.6 cm diameter) in Buffer B with 400 mM KCl.

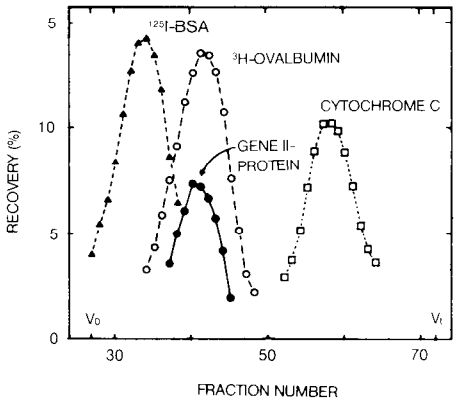

Fig. 2. Gel filtration of gene II-protein. Gene II-protein (14 U, Fraction VII), [^{125}I]-BSA (20,000 cpm), [^{3}H]-ovalbumin (5,000 cpm), cytochrome C (0.5 A_{400}) in 0.4 ml column buffer were loaded on an Ultrogel AcA 44 column (19 ml; 1 cm diameter) in 20% glycerol, 50 mM Tris·Cl (pH 7.5), 5 mM β-mercaptoethanol, 1 mM EDTA, 100 mM NaCl. Fractions were 0.4 ml. The exclusion volume (V_0) and the total volume (V_t) of the column were determined by filtration of Dextran blue and bromophenol blue, respectively.

VII. Hydroxylapatite chromatography: Active fractions (Fraction VII) were applied to a hydroxylapatite column (1 ml; 0.8 cm diameter). The column was washed with 2 ml of Buffer B with 400 mM KCl, then with 2 ml of Buffer B with 10 mM phosphate and the activity was eluted with a 10 ml gradient of Buffer B with 10 mM phosphate to 500 mM phosphate. Fractions of 0.2 ml were collected. The peak fractions were pooled and dialysed against Buffer B with 400 mM KCl containing 50% glycerol and 1 mM EDTA for at least 5 h and stored at -80° (Fraction VII). The activity at this stage was stable for at least one year. The native molecular weight of gene II-protein was determined by gel filtration (Fig. 2) and was the same as found on SDS gels (see main part).

Preparation of phage RFI

Strain Hfr C6 in 6 l tryptone enriched medium was infected with phage fd am 51 (moi = 20) at 5 x 10^8 cells/ml and further grown for 30 min at 30°. The cells were spun down and suspended in 240 ml 50 mM NaCl, 50 mM Tris·Cl (pH 7.5). Then 10 ml 0.5 M EDTA and 50 ml 5 M NaCl were added, followed by 50 ml 10% sodium dodecylsulfate. The lysate was warmed up to 45° in a waterbath and then chilled in ice for 2 h. After centrifugation in a Sorvall GSA rotor for 30 min at 13,000 rpm, residual dodecylsulfate in the supernatant was removed by adding 50 g KCl and clearing by centrifugation. DNA in the supernatant was precipitated with 10% polyethylene glycol 6000, centrifuged and the pellet was dissolved in 50 ml 20 mM Tris·Cl (pH 7.5), 1 mM EDTA and 1% Sarkosyl. To separate RFI from other DNA 50 g CsCl were dissolved in the solution and 3 ml ethidium bromide (10 mg/ml) added. Density banding was carried out in a Sorvall vertical rotor TV 850 or in a Beckman fixed angle rotor 60 Ti for 40 h and 40,000 rpm at 20°. The lower band was collected, rebanded and extracted five times with n-butanol. The DNA solution was dialysed by three changes of 250 ml 20 mM Tris·Cl (pH 7.5), 1 mM EDTA. The yield was 1.1 mg RFI. For special purpose residual RNA was removed from the RF by gel filtration on Bio Gel A-1.5m. The DNA was stored frozen in 20 mM Tris·Cl (pH 7.5), 1 mM EDTA. Five to ten times less fd RFI was obtained from wild type infected cells. Phage øX174 RFI was prepared in an analogue way after infecting strain HF4704 with øX174 am3 mutant (moi = 10). In this case chloramphenicol (30 µg/ml) was added at 10 min after infection.

Preparation of extracts for RF replication

A. Uninfected cells - H570 cells were grown to early log phase in 6 l tryptone enriched medium, spun down and the cell paste was suspended in an equal volume of 10% sucrose, 50 mM Tris·Cl (pH 7.5) and was frozen in liquid nitrogen. The thawed cells were lysed with 200 µg egg white lysozyme added per ml in the presence of 0.1 M NaCl and 10 mM spermidine by incubation for 45 min in ice. The partial lysate was then poured into liquid nitrogen. After thawing, the viscous lysate was spun in a Beckman 50 Ti rotor for 60 min at 50,000 rpm at 0°. Protein in the supernatant was precipitated with 0.25 g ammonium-sulfate added per ml. After keeping for 20 min in ice, the precipitate was spun in a Beckman 50 Ti rotor for 60 min at 50,000 rpm and 0°. The pellet was dissolved in 1.5 ml 10% glycerol, 20 mM Tris·Cl (pH 7.5), 0.2 mM EDTA, 5 mM β-mercaptoethanol, 10 mM NaCl and dialysed against this buffer for 2 h (cell extract).

B. Phage infected cells - Strain H560 was infected with phage fd (wild type or amber mutants; moi = 20) at a density of 5 x 10^8 cells/ml at 37° and grown for 30 min at 30°. In some experiments chloramphenicol (30 µg/ml) was added 10 min after infection. The cell extracts were prepared as described above for uninfected cells.

Exonuclease assay

Phage fd 11 RFI (150 ng), which carries a single Eco RI site, was added to 20 µl buffer containing 50 mM KCl, 20 mM Tris·Cl, pH 7.5, 6 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mM ATP and converted to the linear form with endonuclease Eco RI. At the end of the digestion the sample was heated at 70° for 10 min. Gene II-protein (0.2 units) was added and the mixture incubated for various times at 37°C. The linear DNA was then circularized with phage T4 DNA ligase and a sample added to $CaCl_2$-treated *E.coli* cells (1).

Decrease of 20% for the transfection efficiency were found after 60 min incubation. This is equivalent to about 10^{-5} units of exonuclease III (3) per unit of gene II-protein.

Buffers

Buffer A contained 10% glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol and various concentrations of maleic acid·NH_4^+ (pH 6.8 at 20°) or imidazol·Cl (pH 6.8) and NaCl. Buffer B contained 10% glycerol, 20 mM imidazol·Cl (pH 6.8), 5 mM β-mercaptoethanol, and various concentrations of KCl or potassium-phosphate (pH 6.8).

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Bacteriophage fd gene II-protein. I. Purification, involvement in RF replication, and the expression of gene II.

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