Synthesis of Bacteriophage M13-Specific Proteins in a DNA-Dependent Cell-Free System

II. In Vitro Synthesis of Biologically Active Gene 5 Protein

RUUD N. H. KONINGS, JOSEPHINE JANSEN, THEO CUYPERS, AND JOHN G. G. SCHOENMAKERS

Laboratory of Molecular Biology, University of Nijmegen, Nijmegen, The Netherlands

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It is shown that gene 5 protein of bacteriophage M13 is one of the major proteins synthesized in vitro under the direction of M13 replicative-form DNA. By means of DNA-cellulose chromatography, this protein has been purified to homogeneity and its biological characteristics have been compared with those of its native counterpart. Like native gene 5 protein, the purified, in vitro-synthesized protein binds tightly and selectively to single-stranded, but not to double-stranded, DNAs. These results suggest that truly functional gene 5 protein is made in the cell-free system.

Immediately after penetration of its host cell, the circular single-stranded DNA genome of the male-specific coliphage M13 is converted into a double-stranded "parental" replicative-form (RF) molecule (3, 17, 18; for review see 13, 16). This parental RF molecule is replicated semi-conservatively to produce a pool of progeny RF molecules (7, 12, 15, 19, 20, 28). At a later stage of infection, double-strand synthesis ceases and single-stranded (SS) DNA is produced by an asymmetric replication process in which the viral strand is displaced as a new one is being synthesized (4, 8, 19, 21, 22, 24). For the latter process, an unknown number of host cell functions, in addition to the M13-specific proteins encoded by gene 2 and gene 5, is required. Gene 2 protein is involved in the formation or perhaps stabilization, or both, of a strand-specific discontinuity in the RF-I molecule (7, 12, 17, 28). (RF-I refers to RF DNA in which both strands are covalently closed circles.) Gene 5 protein seems to have a dual function. First, it has a positive role in the asymmetric displacement synthesis of SS DNA and, second, it acts as a repressor by blocking the function of a DNA (RNA?) polymerase which converts intracellular single strands to double-stranded RF molecules (14, 17, 23, 25).

In this paper, the in vitro synthesis of functional gene 5 protein will be demonstrated. This protein has been purified from the in vitro system to homogeneity, and its biological characteristics have been compared with those of native gene 5 protein.

MATERIALS AND METHODS

Bacteria and phages. *Escherichia coli* strain AB301 Hfr (λ) met⁻, RNase I⁻ was used both for the cultivation of wild-type M13 phage as well as for the preparation of the cell-free extracts. *E. coli* K-12 159F⁺, UV⁺, Su⁻ was used for the cultivation of ³⁵S-labeled M13 phages. Bacteriophage M13 wild type was obtained from P. H. Hofschneider.

Media and buffer. The *E. coli* strain AB301 was cultivated in M-9 medium (6). For the preparation of ³⁵S-labeled M13 phages, *E. coli* K-12 159F⁺ was grown in M-9 medium containing MgCl₂ in place of MgSO₄ (29). Buffer A consists of 0.02 M Tris-hydrochloride (pH 8.1), 0.05 M NaCl, 5 mM EDTA, 1 mM β -mercaptoethanol, and 10% glycerol. NET (0.1 M) is 0.01 M Tris-hydrochloride (pH 7.6), 0.1 M NaCl, and 1 mM EDTA. NET (1 M) is 0.01 M Tris-hydrochloride (pH 7.6), 1 M NaCl, and 1 mM EDTA.

Preparation of radioactive viral proteins serving as markers for polyacrylamide gel electrophoresis. The method for the preparation of ³⁵S-labeled phage M13 proteins, gene 3 protein, and gene 8 protein, respectively, has been described previously (10).

¹⁴C-leucine-labeled gene 5 protein was a generous gift from Karl Wanner, Munich.

Preparation of M13 phages and of RF DNA. To prepare large quantities of M13 phages, *E. coli* AB301 was grown with aeration in 10 liters of M-9 medium. At a density of 4×10^8 cells/ml, the culture was infected with phage M13 at a multiplicity of 20. Incubation was continued for 90 min at 37°C. The infected cells were harvested by centrifugation, washed twice with NET (0.1 M), and, after suspension in 250 ml of the same buffer, used for the preparation of RF DNA (see below).

The phages were recovered from the supernatant as described by Yamamoto et al. (30) and finally puri-

fied by two cycles of isopycnic banding in CsCl gradients.

For the preparation of RF DNA, the infected cells were lysed according to the modified Hirt procedure as described by Brutlag et al. (3). After centrifugation for 30 min at $80,000 \times g$, the suspension was extracted twice with an equal volume of phenol saturated with NET (0.1 M). The nucleic acids were precipitated from the aqueous layer by the addition of 0.1 vol of 3 M sodium acetate (pH 5.5) and 2.5 vol of ethanol (-20°C). The precipitate was collected by centrifugation and dissolved in 100 ml of NET (0.1 M). To destroy contaminating RNA, the RF preparation was treated with pancreatic RNase and subsequently phenolized as described by Sugiura et al. (26). After ethanol precipitation, the nucleic acids were dissolved in 20 ml of NET (0.1 M). To remove the degradation products of RNA and other low-molecular-weight material, the solution was passed through a Sephadex G-100 column (5 by 50 cm) which was equilibrated in NET (0.1 M). The material eluting at the void volume was collected and precipitated with ethanol. The nucleic acids were dissolved in 10 ml of NET (1 M), and the solution was brought to pH 11.8 with 1 N NaOH. After 3 min of incubation at 20°C , the solution was neutralized by the addition of 1 N HCl. To remove the SS DNA formed at this denaturation step, the solution was percolated through a nitrocellulose column which was equilibrated in NET (1 M). RF DNA was recovered from the eluate by ethanol precipitation. After standing overnight in the freezer, the precipitate was collected by centrifugation, dried under vacuum, and dissolved in 0.1 M Tris-hydrochloride, pH 7.8. This fraction consisted mainly of RF-I DNA. If required, RF-I free of RF-II (RF DNA with one or more single-strand breaks) was prepared from this fraction by means of preparative sucrose density gradient centrifugation. The yield was 10 to 12 OD_{260} (optical density at 260 nm) units of RF DNA.

Preparation of phage DNA. For the preparation of SS DNA, a suspension of purified virions was mixed at room temperature with 0.1 vol of 10% sodium dodecyl sulfate. After mixing and standing for 1 min at room temperature, an equal volume of phenol saturated with NET (0.1 M) was added. This mixture was shaken continuously over a period of 10 min. The emulsion was then broken by centrifugation for 10 min at $5,000 \times g$. The aqueous top layer was removed and re-extracted twice with phenol saturated with NET (0.1 M). Finally, the aqueous layer was dialyzed against several changes of either 0.1 M Tris-hydrochloride (pH 7.8) or buffer A.

In vitro protein synthesis. The procedure for the preparation of cell-free extracts and the conditions of protein synthesis in vitro have been described (10). After incubation for 40 min at 37°C , to each 100 μl of incubation mixture 25 μl of pancreatic DNase I (0.4 mg/ml) and 375 μl of unlabeled gene 5 protein (0.35 mg/ml) were added. After an additional incubation for 15 min at 37°C , the mixture was dialyzed extensively against three changes of 200 vol of buffer A. Part of the dialyzed solution was used for the analysis of the proteins synthesized, while the remaining part was used for the purification of the in vitro-synthesized gene 5 protein.

Polyacrylamide gel electrophoresis. Polyacrylamide gels, containing 0.1% sodium dodecyl sulfate, were prepared and run essentially as described by Summers et al. (27). The gels included 8 M urea to insure solubility of viral-specific proteins and to improve the separation of small proteins (9, 18). After electrophoresis, the gels were sliced longitudinally and stained with Coomassie brilliant blue. After destaining, the slices were dried under vacuum on filter paper and autoradiographed (5).

Preparation of gene 5 protein. The in vitro-synthesized gene 5 protein was isolated from the incubation mixture by DNA-cellulose chromatography essentially as described by Alberts et al. (1). SS DNA-cellulose was prepared as described by Alberts and Herrick (2). After extensive dialysis, 0.8 ml of the incubation mixture was loaded at 6 ml/h onto a denatured calf thymus DNA-cellulose column (0.5 ml packed volume) which had been equilibrated in buffer A. After rinsing the column with 12 ml of buffer A, the bound proteins were eluted at 6 ml/h in 3-ml steps of 0.15, 0.40, 0.60, and 2.0 M NaCl dissolved in buffer A. Fractions of 0.25 ml were collected, of which 5- μl samples were taken for the determination of the radioactivity. Fractions containing the highest radioactivity were pooled and dialyzed extensively against buffer A. Part of these pooled fractions was used, after the addition of carrier protein, for the analyses of the proteins on polyacrylamide gels, while the remaining part was used to study DNA-protein complex formation.

Because the gene 5 protein already proved to be homogeneous after the DNA-cellulose step, chromatography on DEAE-cellulose columns as described by Alberts et al. (1) was omitted. Similar observations have been made by other investigators (K. Wanner, personal communication).

Analysis of complex formation between gene 5 protein and DNA. Complex formation between gene 5 protein and DNA was carried out as described by Alberts et al. (1). After incubation (20 min at 20°C), the mixtures were layered onto linear sucrose gradients (5 to 30%) prepared in buffer A. After centrifugation for 3 h at 50,000 rpm in a Spinco SW65 rotor at 4°C , the gradients were displaced by a heavy sucrose solution (65%) pumped through a needle, the outlet of which carefully was placed at the bottom of the gradient. The optical density was monitored with the aid of a LKB optical unit coupled to a logarithmic recorder. Two-drop fractions were collected into scintillation vials. After addition of 0.35 ml of water and 5 ml of toluene-based scintillation mixture containing 0.4% Omnifluor and 25% (vol/vol) Triton X-100, the samples were counted in a Packard liquid scintillation counter.

RESULTS

In vitro synthesis of gene 5 protein. In a previous communication (10), we have shown the effect of addition of M13 RF-I DNA to a DNA-dependent cell-free system of *E. coli*. From these results it could be concluded that RF-I DNA gives rise to the synthesis of at least seven proteins (I through VII), ranging in mo-

lecular weight from 5,000 to 70,000, which had no equivalent protein synthesized in the endogenous system. Two of these proteins (VI and VII) co-migrated on polyacrylamide gels with the native proteins encoded by genes 5 and 8, respectively. Furthermore, these proteins also were synthesized in much larger quantities than the other phage-specific proteins. Evidence that the *in vitro* protein VI was identical to native gene 5 protein was derived from protein synthesis experiments involving the use of ^{14}C -tryptophan, an amino acid not present in gene 5 protein (14; unpublished data). When this amino acid was used to label the proteins, no synthesis of a low-molecular-weight protein co-migrating with native gene 5 protein could be demonstrated.

Purification of the *in vitro*-synthesized gene 5 protein. In order to obtain evidence of whether the *in vitro*-synthesized gene 5 protein was also truly functional, experiments were performed to compare the biological characteristics of this *in vitro* product with those of its native counterpart. In Fig. 1 the chromatography results of the different incubation mixtures on single-stranded DNA-cellulose columns are presented. It can be concluded that only in the incubation mixture to which M13 RF-I DNA had been added were proteins synthesized which were specifically retained by the DNA-cellulose column. These proteins started to elute at an NaCl concentration of 0.4 M, and elution was complete after the addition of 0.6 M NaCl. Although the proteins did not elute from the column as one well-defined peak, analyses by polyacrylamide gel electrophoresis revealed that they consisted of electrophoretically pure gene 5 protein (Fig. 2B, f-h). No contamination with other proteins could be detected, even after extended exposure times. Comparison of the

FIG. 1. DNA-cellulose chromatography of the proteins synthesized in the cell-free system of *E. coli* in the absence and presence of phage M13 RF-I DNA. Symbols: ●, elution pattern of ^{14}C -labeled proteins synthesized *in vitro* in the absence of phage M13 RF-I DNA; ○, elution pattern of ^{14}C -labeled proteins synthesized *in vitro* in the presence of phage M13 RF-I DNA. The proteins were labeled with ^{14}C -arginine, an amino acid not present in gene 8 coat protein (10). After loading, the column was washed with 12 ml of buffer A, and the bound proteins were then eluted by increasing the NaCl concentration in the column buffer as indicated. Fractions of 0.25 ml were collected, of which 5-μl samples were taken for the determination of radioactivity. Peak fractions were pooled as indicated and dialyzed against buffer A. Part of these pooled fractions was used, after the addition of chymotrypsinogen A (molecular weight 25,700) as a carrier for the CCl_3COOH -precipitation, for the analysis of the proteins on SDS-polyacrylamide gels, while the remaining part was used to study DNA-protein complex formation.

position of elution of the *in vitro*-synthesized gene 5 protein (Fig. 2B, f-h) with that of its native counterpart (Fig. 2A, e-g) strongly indicates that their chromatographic behavior on

FIG. 2. Electrophoretic analysis on SDS-polyacrylamide gels of the protein fractions obtained after DNA-cellulose chromatography (Fig. 1). A, Photographs of gel slices stained with Coomassie blue: a, b, proteins present in the incubation mixture, supplemented with native gene 5 protein, before being applied to the column. a, The mixture with M13 RF-I DNA; b, the mixture without M13 RF-I DNA; c, d, proteins present in the incubation mixture and not retained by the column (Fig. 1, peak fractions B and A, respectively). e, f, g, Proteins present in the incubation mixture and retained by the column (Fig. 1, peak fractions I, II, and III, respectively). B, Autoradiographs of dried gel slices: a, phage M13 marker proteins, gene 3 protein (molecular weight 70,000), and gene 5 protein (molecular weight 10,000); b, c, proteins synthesized *in vitro* in the presence (b) and absence (c) of phage M13 RF-I DNA, before they were applied to the column; d, e, proteins synthesized *in vitro* in the presence and absence of phage M13 RF-I DNA and not retained by the column (Fig. 1, peak fractions B and A, respectively); f, g, h, proteins synthesized in the presence of phage M13 RF-I DNA and retained by the column (Fig. 1, peak fractions I, II, and III, respectively). As described in the Materials and Methods section, native unlabeled gene 5 protein was added to the incubation mixtures before being applied to the column. As a carrier for the CCl_3COOH -precipitation, 15 μg of chymotrypsinogen A (molecular weight 25,700) was added to the peak fractions I, II, and III (Fig. 1) before analysis on SDS-polyacrylamide gels. The letters a through g on top of each column in A correspond to the letters b through h on top of each column in B. The Roman numerals I through VII indicate the position of migration of the proteins synthesized in the presence of M13 RF-I DNA and which have no equivalent protein synthesized in the endogenous system. As, in the experiments described here, the proteins have been labeled *in vitro* with ^{14}C -arginine, an amino acid not present in gene 8 coat protein (molecular weight 5,200), no protein migrating on position VII could be demonstrated.

DNA-cellulose columns is identical. The fact that gene 5 protein did not elute from the DNA-cellulose column as a single, well-defined peak can probably be explained by the cooperativity of the DNA binding, since it has been observed that the position of elution of gene 5 protein is dependent on its concentration in the extract (see ref. 1; unpublished data).

Furthermore, it can be concluded from the results obtained that about 13% of the incorporated radioactivity was present in gene 5 protein. This percentage is an underestimation since, in contrast to native gene 5 proteins (Fig. 2A, a-d), not all of the in vitro-synthesized gene 5 protein was retained by the DNA-cellulose column (Fig. 2B, b and d). However, as judged from the intensities of the gene 5 protein bands in comparison with those of the other in vitro-synthesized proteins (Fig. 2B, b and d), the amount not retained by the column constitutes only a minor part of the gene 5 protein synthesized in vitro.

Binding of the in vitro-synthesized gene 5 protein to DNA. One of the main functions of native gene 5 protein is to protect SS DNA molecules from being converted back into double-stranded RF DNA before they can be packed into mature phage particles (17, 23, 25). The observations that in the infected cell the number of gene 5 protein molecules is strikingly larger than that of progeny SS DNA molecules suggests that this protein serves a structural rather than a catalytic role in DNA replication (1, 9, 14, 20). This suggestion is supported by the recent findings that one of the main characteristics of biologically active gene 5 protein is that it binds tightly and cooperatively to single-stranded but not to double-stranded DNA (1, 14). In order to obtain information about whether the in vitro-synthesized gene 5 protein had similar characteristics, binding studies on single- and double-stranded DNAs were performed. From the results presented in Fig. 3, it can be concluded that the purified gene 5

Fig. 3. Binding of the in vitro-synthesized gene 5 protein to DNA. DNA and gene 5 protein were mixed in 0.2 ml of buffer A. After 20 min of incubation at room temperature, the mixtures were sedimented through a linear sucrose gradient (5 to 30%) prepared in buffer A. Symbols: —, optical density at 254 nm; ●, radioactivity. A, 1,200 counts/min of gene 5 protein (15 μ g); B, 1,200 counts/min of gene 5 protein (15 μ g) and 15 μ g of phage M13 SS DNA; C, 1,200 counts/min of gene 5 protein (15 μ g) and 15 μ g of phage M13 RF DNA.

protein binds tightly and selectively to single-stranded M13 DNA but not to double-stranded M13 RF DNA. Comparable results were obtained when DNA-protein complex formation was studied in the presence of either single- or double-stranded phage T3 DNA (unpublished data).

DISCUSSION

It is clear from the results presented here and elsewhere (10) that phage M13 RF-I DNA directs in the cell-free system of *E. coli* the synthesis of at least the proteins encoded by genes 5 and 8. As has been observed in vivo (9), these two proteins are also synthesized in vitro in much larger quantities than are the other phage-encoded proteins. From the results obtained, it furthermore can be concluded that approximately 13% of the incorporated radioactivity is present in gene 5 protein. This percentage allows us to calculate that in the in vitro system about 15 gene 5 protein molecules are synthesized per RF-I molecule. The latter value is probably much lower than the number of gene 5 protein molecules synthesized in the infected cell per RF-I molecule (cf. ref. 1, 9, 14, 20). However, the overall similarities between the results obtained from both protein-synthesizing systems suggest that in both systems the same regulatory mechanism(s) is operative which insures that gene 5 and gene 8 protein are synthesized more frequently than are the other phage-encoded proteins. The techniques described here may offer a valuable tool for studying the regulatory mechanism(s) (cf. 10) which are responsible for this diversity in gene expression.

The in vitro-synthesized gene 5 protein has an identical chromatographic behavior on SS DNA-cellulose columns as its native counterpart. Furthermore, both proteins bind tightly and selectively to single-stranded but not to double-stranded DNAs. Since the capacity to form selectively a complex with SS DNA seems to be the main characteristic of biologically active gene 5 protein (1, 14, 23, 25), these data strongly indicate that the biological characteristics of native and in vitro-synthesized gene 5 protein are identical. The conclusion therefore seems to be justified that in the cell-free system truly functional gene 5 protein is made.

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