Overexpression, Purification, and Characterization of Escherichia coli Bacteriophage PRD1 DNA Polymerase

IN VITRO SYNTHESIS OF FULL-LENGTH PRD1 DNA WITH PURIFIED PROTEINS*

(Received for publication, February 26, 1991)

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The bacteriophage PRD1 DNA polymerase gene (gene I) has been cloned into the expression vector pPLH101 under the control of the λ pI promoter. Tailoring of an efficient ribosome binding site in front of the gene by polymerase chain reaction led to a high level heat-inducible expression of the corresponding gene product (P7) in Escherichia coli cells. Expression was confirmed in vivo by complementation of phage PRD1 DNA polymerase gene mutants and in vitro by formation of the genome terminal protein P8-dGMP replication initiation complex. Expressed PRD1 DNA polymerase was purified to apparent homogeneity in an active form. DNA polymerase, 3'-5'-exonuclease, and P8-dGMP replication initiation complex formation activities cosedimented in glycerol gradient with a protein of 65 kDa, the size expected for PRD1 DNA polymerase. The DNA polymerase was active on DNase I-activated calf thymus DNA, poly(dA)·oligo(dT) and poly(dA·dT) primer/templates as well as on native phage PRD1 genome. The 3'-5' exonuclease activity was specific for single-stranded DNA and released mononucleotides. No 5'-3' exonuclease activity was detected. The inhibitor/activator spectrum of the PRD1 DNA polymerase was also studied. An in vitro replication system with purified components for bacteriophage PRD1 was established. Formation of the P8-dGMP replication initiation complex was a prerequisite for phage DNA replication, which proceeded from the initiation complex and yielded genome length replication products.

Bacteriophage PRD1 belongs to a close family of lipodContaining dsDNA bacteriophages infecting Gram-negative bacteria harboring F, N, or W incompatibility group plasmids. Among the hosts are Escherichia coli and Salmonella typhimurium (Olsen et al., 1974; Bamford et al., 1981). The genome is a linear molecule of about 15.0 kb (Bamford et al., 1991). At both genome ends there are 110 bp long inverted terminal repeats (Savilahiti and Bamford, 1986) and 5'-covalently linked terminal proteins (Bamford et al., 1983). The PRD1 system is reviewed in more detail by Mindich and Bamford (1988) and Caldentey et al. (1990).

The left genome end codes for two very early proteins (Mindich et al., 1982; McGraw et al., 1983; Savilahiti and Bamford, 1987; Hsieh et al., 1987; Jung et al., 1987), the genome terminal protein (gene VIII, protein P8) and the DNA polymerase (gene I, protein P1), in this order from the genome terminus. The function of the terminal proteins is to serve as a primer in the initiation of DNA replication (Bamford and Mindich, 1984; Savilahiti et al., 1989). In this event the phage-encoded DNA polymerase catalyzes the linking of dGMP into the Tyr-190 residue in the terminal protein (Hsieh et al., 1990). DNA replication system with cell extracts has indicated that replication can start from either end of the molecule and that terminal protein and DNA polymerase are the only phage-encoded proteins needed to synthesize the full-length phage genome in vitro (Yoo and Ito, 1989).

Based on amino acid sequence comparison, PRD1 DNA polymerase can be classified to the family of eukaryotic a-like DNA polymerases (Savilahiti and Bamford, 1987; Jung et al., 1987; Bernad et al., 1987; Wong et al., 1988). A special feature of the enzyme is its capability of initiating the DNA replication by a protein priming mechanism (reviewed by Salas (1988a, 1988b)). This type of mechanism is also used by the DNA polymerases from the extensively studied linear DNA replication systems of adenovirus (Horwitz, 1986; Tamanino, 1986; Kelly et al., 1988) and bacteriophage φ29 (Salas, 1988b).

The time course of the expression of the early proteins in PRD1-infected cells suggests that the expression of the phage genome terminal protein and the DNA polymerase are transcriptionally coupled (Mindich et al., 1982). This is consistent with the finding that no obvious promoter precedes the DNA polymerase gene (Savilahiti and Bamford, 1987).
The DNA polymerase gene is expressed at a much lower level than the terminal protein gene. The low expression is suggested to result either from the lack of a RBS leading to a coupled translation to gene VIII or from an inefficient RBS in front of gene I (Savilahti and Bamford, 1987).

One line of research in our laboratory is aimed toward understanding of the replication mechanism of PRD1 genome, the only protein primed replication system known to operate in E. coli. As a first step, we are characterizing the products of all of the phage genes that are involved in this process. We report here the overexpression, purification, and characterization of the bacteriophage PRD1 DNA polymerase. The enzyme was shown to possess DNA polymerase activity and protein-priming activity, as well as 3'→5'-exonuclease activity. Replication of bacteriophage PRD1 DNA was achieved with purified proteins. Accordingly, this is the first time that a protein-primed DNA replication system originally operating in E. coli is available with purified components in vitro.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

We constructed recombinant plasmids, where the bacteriophage PRD1 DNA polymerase gene (gene I) was under the control of the λ pol promoter. Because plasmid pUSH10 was able to complement PRD1 gene I mutant phages and, in addition, extracts of strains harboring this plasmid were active in the protein-priming assay, we conclude that there is a functional phage-derived RBS in front of gene I. However, the expression could not readily be detected by SDS-PAGE. Downstream of gene I, plasmid pUSH10 contained also a 5' terminal portion of the PRD1 gene XV preceded by its original phage RBS. This truncated gene was highly expressed allowing the detection of its gene product by SDS-PAGE.

To increase the gene I expression, we tailored an efficient RBS in front of the gene. For this purpose, we applied extended primer PCR methodology. Successful use of a similar strategy in plasmid constructions and cloning has been described (Vallette et al. 1989; MacFerrin et al. 1990). The plasmid with changed RBS in front of gene I (pUSH100) directed high level expression of P1 which could be visualized by SDS-PAGE (Fig. 2). Even though the P1 expression was increased at least 100-fold compared with that from plasmid pUSH10, the expression of gene XV fragment was the same from both plasmids. This indicated that the changes made in front of gene I were responsible for the increased expression of this gene. Unfortunately most of the overexpressed P1 and the N-terminal polypeptide coded by the truncated gene XV aggregated into an insoluble form. Nevertheless, the amount of the soluble form of P1 produced with plasmid pUSH100 was considerably higher than that produced with plasmid pUSH10. In an attempt to purify the aggregated material, the polypeptides copurified showing the heterologous composition of the aggregate (data not shown). To overcome the aggregation problem, gene XV from plasmid pUSH10 was deleted, and in addition, the induction temperature was lowered. Neither approach, however, helped to solve this problem. Therefore, purification of the small, soluble fraction of the protein was carried out. Plasmid pUSH110 was chosen to express P1 for purification because it contained the whole gene I but only a small portion of gene XV.

**Cosedimentation of DNA polymerases, 3'→5'-exonuclease, and protein-priming activities with P1 indicated that it was responsible for all these activities. The other well-characterized DNA polymerases from protein-primed DNA replication systems, adenovirus (Field et al., 1984) and bacteriophage ø29 of Bacillus subtilis (Blanco and Salas, 1984, 1985a), also have similar activities present in the single polypeptide. This might be a general feature of DNA polymerases capable of protein priming. Native PRD1 genome served as a template for P1 when primed with terminal protein P8. The enzyme could also use a variety of DNA primer/templates such as poly(dA)-oligo(dT), poly(dA-dT), and activated DNA. In the latter cases the PRD1 DNA polymerase activity catalyzed the incorporation of nucleotides from a DNA primer. These priming properties of P1, together with its 3'→5'-exonuclease activity are in agreement with the generally accepted view of replication of DNA molecules with covalently linked terminal proteins (Salas, 1988b).

Originally based on drug sensitivity, and later on amino acid sequence analysis, DNA polymerases have been divided into two groups: eukaryotic α-like DNA polymerases and E. coli polymerase I-like DNA polymerases (Bernad et al., 1987; Jung et al., 1987; Wong et al., 1988). α-like DNA polymerases have been reported to be sensitive to the drug aphidicolin and to certain nucleotide analogues such as BuvATP and BuvDTP (Bernad et al., 1987). Here we have demonstrated the sensitivity of PRD1 DNA polymerase to these compounds. In our hands, however, Klenow enzyme was equally or more sensitive to these drugs with poly(dA)-dT primer/template. The reason for this remains unknown. The drug sensitivities of DNA polymerases may, however, depend also on reaction conditions. A more reliable basis for classification of DNA polymerases is certainly the amino acid sequence data, which verifies that PRD1 DNA polymerase belongs to α-like DNA polymerases (Savilahti and Bamford, 1987; Bernad et al., 1987). The higher sensitivity of PRD1 DNA polymerase to NEM as well as its activation by dimethyl sulfoxide clearly distinguished it from Klenow enzyme. The latter observation might later be helpful in establishing a simple and specific assay for purification of this protein. PRD1 DNA polymerase contained 3'→5'-exonuclease activity, which acted on single-stranded DNA and released mononucleotides. The same properties have been found to be associated with bacteriophage

![Fig. 8. DNA polymerase activity of protein P1 with PRD1 DNA-P8 as a template.](image-url)
polymerase (Pr), and 4 pg of PRD1 DNA-protein P8. After incubation at 37 °C for 5, 10, 20, and 30 min, respectively; lane h, protease-treated PRD1 genome (1 µg) stained with EtBr as a control.

It has been shown earlier that bacteriophage PRD1 uses a protein-priming mechanism to initiate its replication. In this event the phage DNA polymerase catalyzes the linking of dGMP to the Tyr-190 residue in the terminal protein (Bamford and Mindich, 1984; Hsieh et al., 1990). Full-length PRD1 genome replication in vitro has been described earlier using cell extract which contained both PRD1 DNA polymerase and terminal protein (Yoo and Ito, 1989). However, in this case the interpretation of the results does not rule out the participation of host factors in replication. We have shown here that two purified proteins, DNA polymerase and terminal protein, were sufficient to replicate the PRD1 genome in vivo when intact PRD1 DNA-protein P8 template was used (Fig. 8). This in vitro replication yielded full-length products in 10 min (Fig. 9). The rate of elongation at 37 °C was approximately 25 nucleotides/s, which is close to that obtained in the φ29 system (Blanco and Salas, 1985b).

Bacteriophage φ29 DNA polymerase has been shown to replicate the phage DNA by a strand displacement mechanism supporting the symmetric model of DNA replication, where the replication can start from either terminus of the molecule (Blanco et al., 1989). In analogy to the φ29 system, together with the fact that PRD1 replication can start from both ends of the molecule (Yoo and Ito, 1989), we assume that the mechanism of PRD1 replication is similar. First protein priming takes place, after which DNA polymerase continues elongation simultaneously displacing the parental strand of the same polarity.

Ammonium ions stimulate the initiation of PRD1 DNA replication in vitro (Savilahti et al., 1989). The elongation on poly(dA)-oligo(dT) primer/template in this study, however, was not stimulated by ammonium indicating the specificity of stimulation to the initiation step. Similar results have been achieved in the bacteriophage φ29 system, where the stimulation is specifically due to the stabilization of the complex between the soluble terminal protein and DNA polymerase prior to the initiation of replication (Blanco et al., 1987). The specific mechanism in the PRD1 system is still unknown but might well be similar to that of φ29.

The availability of this in vitro DNA replication system and a high frequency electroporation system (Lyra et al., 1991) for PRD1 genome allows the introduction of the in vitro produced molecules into the cell to test their biological activity. Furthermore, since many DNA replication mutants of E. coli and purified replication proteins are available, the PRD1 system offers a good opportunity to study the participation of possible host factors in protein-primed DNA replication in general.

Acknowledgments—We want to thank Sisko Litmanen and Merja Nissinen for their skillful technical assistance. Tapio Kesti is acknowledged for preparing different primer/templates and for helpful discussions. Dr. G. E. Wright is appreciated for supplying the nucleotide analogues BuAdATP and BuDGTP.

REFERENCES

**Bacteriophage PRD1 DNA Polymerase**

**EXPERIMENTAL PROCEDURES**

**Materials**

Nucleotides and enzymes


**Preparation and cell extracts**

Bacteriophage PRD1 genomic terminal prokaryotes (967) was purified as described by Salas et al. (1989). Complementing cell extract for protein-genome assay was prepared from E. coli strain W3110 41M130 by using heat induction as described by Maniatis et al. (1982). Salas et al. (1989). Cells extracts for analysis of protein were prepared from cells carrying different recombinant plasmids as described by Salas et al. (1989) for strain 16M16 (1980). The protein concentration of each extract was assayed by adjusting to approximately 0.3 mg/ml.

**Primer/template for polymerase assay**

Bacteriophage PRD1 genome was isolated by phenol extraction without proteinase treatment as described by Salas et al. (1991). DNA was propagated in E. coli strain W3110 41M130 and was extracted as described by Salas et al. (1989). Denatured calf thymus DNA was prepared by heating for 5 min at 90°C and rapid cooling on ice. Poly(rA)poly(rT) was prepared by incubating 100 ng of DNA with 400 units of ribonuclease-free DNA polymerase from Escherichia coli 1.2.4 and 0.5 units of Escherichia coli 1.2.6 and 0.5 units of ribonuclease-free DNA polymerase from Escherichia coli 1.2.4.

**Sucrose gradients, bacteirhages and plaque assay**

Plasmid p1C18 (vector [Franco-Perez et al. 1989] and plasmid p1C18 [Bouguen et al. 1983] containing the insulin promoter were propagated in E. coli strain W3110 41M130. Plasmid p1C18 (vector [Franco-Perez et al. 1989] and plasmid p1C18 [Bouguen et al. 1983] containing the insulin promoter were propagated in E. coli strain W3110 41M130. The expression vector p1C18 was propagated in E. coli strain W3110 41M130.

**Acknowledgments**

The author would like to thank Dr. M. Sargent, Department of Medical Chemistry, University of Helsinki for helpful advice and assistance. This work was supported by a grant from the Academy of Finland.
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**Methods**

Construction of the expression plasmids pUSH10, pUSH100 and pUSH110.

The strategy for construction of the expression plasmids is shown in Fig. 1. The recombinant M13mp18 (Yannis Perron et al., 1985) clone M13DSS containing the 197 bp EcoRI fragment of PRDI genome (cloned in the Hinf I site (Sambrook and Maniatis 1987) was cleaved with EcoRI and Hinf. The 2037 bp fragment obtained was ligated into pUSH110 digested with the same two enzymes.

The high level expression plasmids pUSH100 and pUSH110 were constructed as follows:

The plasmid pUSH10 was cleaved with EcoRI. Part of this linearized plasmid DNA was amplified using PCR with synthetic oligonucleotide primers (Fig. 2). The PCR products were extracted with phenol and concentrated by precipitation with ethanol. DNA fragments obtained were digested with EcoRI and PstI and ligated back into the EcoRI-PstI digested expression vector pUSH10 giving rise to plasmid pUSH100. Plasmid pUSH110 was constructed by cleaving pUSH100 with HinfI and subsequently deleting it with XhoI. The entire insert to obtain a high expression efficiency as well as a maximum repression of the E. coli enzymes. Ligations mixtures were transformed into DH5a (p8586), plated and incubated at 26°C.

To sequence the amplified and cloned 2.1 kb EcoRI-PstI fragment restriction fragment the shotgun strategy was applied. This fragment was cleaved separately with restriction enzymes 4bp. Hinf, Hpal, Hpal and BssI. The fragments obtained were filled in with Klenow enzyme and blunt end ligated into the plasmid pUC18 digested with SmaI. The entire sequence was obtained from overlapping clones. Standard DNA manipulations were performed as described by Maniatis et al. (1982). The verification of the relevant DNA sequences was obtained by the plasmid sequencing method following the United States Biochemicals Sequenase protocol with [3"S]ATP and modified T7 DNA polymerase (Faber and Richardson 1987).

**Figure 1. Plasmid constructions**

For details see the text. An asterisk in pUSH100 and pUSH110 indicates the location of the targeted RHS.

**Figure 2. PCR strategy and expression of P1 from recombinant plasmids**

Panel A. Strategy for tailoring an efficient RBS in front of gene I. For details see the text. An asterisk indicates the location of the new RHS.

Panel B. Total cellular proteins of E. coli cells harboring recombinant plasmids analyzed after 3 h heat induction at 42°C as described in the text by a 15% SDS-PAGE. Lanes: A: DH5a (p8587, pUSH1001) negative control B: DH5a (p8587, pUSH1100) C: DH5a (p8587, pUSH1000). An asterisk indicates the position of protein P1. A double asterisk indicates the position of an N-terminal peptide from protein P15 (the gene of which follows gene 1) reading frame in plasmids pUSH100 and pUSH1000. Arrows on the right indicate the positions of bacteriophage 46 proteins (Minkley and Bamford 1988) as molecular weight markers (in kDa).
Protein concentrations were determined according to Bradford (1976) with BSA as standard. Unless otherwise indicated, SDS-polyacrylamide gel electrophoresis was carried out in a Mini-Gel apparatus from Pharmacia (Uppsala, Sweden) as described (Laemmli, 1970). Silver staining procedure was that of Wray et al. (1981). Alkaline agarose gel electrophoresis was performed as described (Prun and the 1988, PAGE of DNA and nucleotides was carried out according to Sampson et al. (1989) in the buffer containing 60 mM Tris-HCL (pH 8.0) and 2 mM EDTA.

**TABLE I. Complementation analysis**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
<th>Type 4</th>
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Expression of the PRDI DNA polymerase gene:
To detect the biological activity of the expressed protein P1 and to verify the plasmid constructions, complementation analysis was carried out with plasmid PRDI DNA polymerase gene mutants and a wild-type strain. Plasmid PM2 was first introduced into the strains used in the analysis. The titer of the wild type and the mutant phenotypes were determined on cells containing the expression plasmids as well as on the control cells containing the vector at 26°C, 31°C and 37°C. Mutant plaque suppression and the difference between wild-type and E. coli plate growth were measured at 37°C using S. typhimurium strains JPS45 (m2) and P2123 (m2) and E. coli strain P4684 (m2). The results are shown in Table I. The expression plasmids pSF1100 and pSF110 were partially complemented both to the P1 suppression mutants already at 26°C. Elevation of the temperature to 37°C led to suppression level complementation of these mutants. Consequently efficiency with plasmid pSF1100 was approximately two fold when compared to that with pSF110.

Protein synthesis from the leader peptide of the expressed plasmid can be switched on in E. coli strains P4684 and DH5α[pSF57] by raising the temperature from 28°C to 42°C. We found that the heat-induced expression of P1 from recombinant plasmids by whole cell SDS-PAGE, and quantitatively, by protein activity assay with cell extracts. Plasmid P8110 transformed E. coli control did not express any protein but expression was obtained from recombinant plasmids pSF110 and pSF1100 (Fig. 2, panel III). The expression from pSF1100 was at least 100-fold when compared to that from pSF110. However, most of the overexpressed protein aggregated and was not available for purification in a soluble form. The protein priming activity in cell extracts was a measure of the amount of the soluble form of P1. An extract from cells carrying plasmid pSF1100 contained approximately eightfold right protein priming activity when compared to that from cells carrying plasmid pSF110 (data not shown). Plasmid pSF1100 and pSF110 expressed roughly equal amounts of P1 and the amount of soluble P1 was similar in both cases (data not shown).

**TABLE II. Purification of bacteriophage PRDI DNA polymerase**

<table>
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<th>Volume</th>
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<td>I</td>
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**RESULTS**

**Construction of the expression plasmids**

The left very early region of the PRDI genome contains the phage DNA polymerase gene, the ORF of which is located within the 1976 bp Hfr restriction fragment. The same fragment is present in the plasmid pM3 (McGraw et al. 1983, Bandlow and McDow 1984). This fragment from plM3 was transferred into MI3M18 sequencing vector as previously described (Sambrook and Russell. 1989). From this construction, using directed cloning, it was possible to move gene I into the expression vector pJ1H101, where it was in the right orientation under the A, promoter pJ1H101. We used the ICE technique to amplify DNA fragments from the plasmid pJS10 in order to tailor an efficient RESS in front of the PRDI gene (Fig. 2). The designed RESS sequence was acquired from the beginning of the PRDI gene XIX (Fukada et al. 1989). This gene has been shown to be highly expressed during the early stages of phage infection (Mondel et al. 1982). The left hand primer contained also an EcoRI restriction site in addition to the tailored RESS (Fig. 2). The amplified product was cloned back into pJ1H101, thus obtaining pJ1H100. The joining regions were analyzed by sequencing and shown to be as predicted. The recombinant plasmid pJ1H100 contained the PRDI gene I under the A, promoter with an efficient RESS in front of the gene. After amplification and cloning, the entire amplified gene was sequenced to detect possible mutations. Except for the tailored RESS, no nucleotide changes were introduced during construction. Since plasmid pSF1100 contained also a region of the PRDI gene AV, was further deleted with exonuclease III. The obtained plasmid pSF1100 was used to express protein P1 for purification.
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Figure 6. Effects of different compounds on polymerase activities of P1 and Klenow enzyme

DNA polymerase assays were conducted as described in 'Methods' with poly(dA)·oligo(dT) as a primer/template at a concentration of 40 μM. Different compounds were added at the concentrations indicated. Panel A. Relative DNA polymerase activities of P1 in the presence of KC1 (●), NaCl (◆) and NH4Cl (▲). Panel B-F. DNA polymerase activities of P1 (●) and Klenow enzyme (◆) in the presence of different inhibitors/activators shown in the panel.

Figure 7. Directionality of the exonuclease activity

Linearized plasmid pUC18 was labelled at the 3' or 5' terminus as described in 'Methods'. Proteins P1 40-3 ng was incubated at conditions described in 'Methods' for the times indicated with native (○) or heat denatured (▲) labelled pUC18 DNA. As controls, heat denatured pUC18 DNA were also incubated without protein P1 (◆). Remaining radioactivity was measured as described in 'Methods'. Panel A. 3' labelled pUC18 (60 000 cpm, 250 ng DNA) as a substrate. Panel B. 5' labelled pUC18 (25 000 cpm, 250 ng DNA) as a substrate. After incubation, samples from reactions containing denatured 3'- or 5' labelled DNA were also subjected to alkaline agarose gel electrophoresis. Panel C. 3' labelled pUC18 as a substrate. Panel D. 5' labelled pUC18 as a substrate. The arrow indicates the migration of linearised pUC18 DNA. On the right are indicated the migration of DNA size markers (in kilobase pairs).

Figure 4. Coordinatiation of DNA polymerase, exonuclease and protein-prining activities with P1 in glycerol gradient centrifugation

A sample of 200 μl of 0.4 μg protein from fraction number 31 of heparin-agarose chromotography was layered onto a 5 to linear glycerol gradient (25:45% v/v) in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 10 mM MgCl2, 0.05% bromophenol blue, and centrifuged in a Beckman SW 50.1 rotor for 14 hours at 50,000 rpm. Fractons of 300 μl were collected from the surface. Molecular weight markers bovine insulin, 43.000 Da, BSA, 68.000 Da, kallikrein, 150.000 Da were separately centrifuged in parallel gradients and their positions are indicated by arrows in panel A. DNA polymerase (panel A, ●), exonuclease (panel A, ▲) and protein-prining (panel C) activities of each fraction were assayed as described in 'Methods'. A sample (10 μl) of each fraction was also analysed by 15% SDS-PAGE stained with silver (panel B). Molecular weight markers were those described in Fig. 3.

Figure 5. Primer/template preferences of P1

The ability of P1 to use different primer/templates was studied by measuring the incorporation of nucleotides as described in 'Methods'. The concentration of each primer/template was 40 μM and for PRD1 DNA-protein P1 20 μg/ml. [●] PRD1 DNA-protein P1, [◆] poly(dA)·oligo(dT), [▲] native calf thymus DNA, [▲] heat denatured calf thymus DNA.

Purification of protein P1:
Purification of P1 was performed from 120 g (wet weight) of E. coli DH5α[pEI57, pUS1106] cells. Purification was followed with DNA polymerase and protein-prining assays and monitored by SDS-PAGE. The purification procedure including four chromatographic steps is described in 'Methods'. The results of purification are summarised in Fig. 3 and in Table I. The contaminating host DNA polymerase activities were eliminated in DEAE-Sepharose and phosphocellulose chromatography. The last step of the purification, heparin-agarose chromatography, yielded essentially pure 65 kDa protein P1 preparation (Fig. 3). The total yield of purified protein P1 from 120 g of cells (heparin-agarose fractions 28-34) was approximately 130 μg.

Three activities of protein P1:
We purified bacteriophage PRD1 DNA polymerase by assaying DNA polymerase activity with activated calf thymus DNA and by protein-prining assay. To ascribe these activities to the purified polypeptide, a sample from the heparin-agarose chromatography was further analysed by glycerol gradient centrifugation (Fig. 4). DNA polymerase and nuclear activity (panel A) as well as protein-prining activity (panel C) coeluted with a 65 kDa protein (panel B). No additional protein bands were detected in the SDS-polyacrylamide gel. These results verified that the 45 kDa protein was P1 and that it was responsible for all three activities measured.
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3'-5' exonuclease activity.

To further characterize the nuclease activity detected in the cosmid preparation experiment, we carried out the exonuclease directionality analysis. Linearized plasmid pUC18 was labeled with "P either at the 3'- or 5'-termini. Purified protein P1 was incubated with these molecules and remaining as well as released radioactivity was analyzed as described in "Methods". Radioactivity was released from the 3'-termini of heat denatured DNA but not from the 3'-termini of native DNA (Fig. 7, panel A), indicating that the nuclease activity was specific for ssDNA. In contrast experiments, (P1 labeled) radioactivity was not released from the 3'-termini of ssDNA. No release of radioactivity was detected from the 3'-termini of either native or heat denatured DNA (panel B).

The release of radioactivity from the 3'-termini of the heat denatured DNA was also demonstrated by alkaline agarose gel electrophoresis (Fig. 7, panel C). The size of the radioactive band remained the same even though the amount of radioactivity decreased. As expected, no release of radioactivity from the 5'-termini was detected (panel D). Furthermore, there was no detectable change in the size of the labeled pUC18 DNA. This was expected, since plasmid pUC18 is 2.4 kbp long and only a considerable nucleotide release from the 3'-terminus could have been visualized as a shortening of the radioactive labelled molecules in this assay.

The released radioactive products from heat denatured 3'-labelled pUC18 DNA were further analyzed by 20% PAGE (data not shown) as described in "Methods". For comparison, the release experiment was also conducted with Klenow enzyme. As internal standards we used inorganic phosphate (P) and 32P-PATP. Since we labeled single-stranded pUC18 with [32P]dCTP, the terminal nucleotide released by Klenow enzyme was dCMP. Radioactive nucleotides released by protein P1 migrated as those released by Klenow enzyme. The obvious conclusion is that P1 released mononucleotides from the 3'-termini of the linearized single-stranded pUC18 DNA. Exonuclease experiments with P1DNA showed that P1 can convert all radioactivity to acid soluble form (data not shown). This verified the successful nuclease release by P1.

Replication of bacteriophage PRD1 genome with purified components.

As described in "Methods" bacteriophage PRD1 DNA replication with P1 was studied using PRD1 DNA (denatured PRD1 genome) as a template, genome terminal protein P8 as a primer and all four nucleotides as substrates (Fig. 8). When all the components were present, accumulation of TCA insoluble radioactivity was detected (panel A). If protein P8 treated PRD1 genome was used as a template, or P1 or P8 were omitted, no accumulation of radioactivity was detected (panels B, C, and D, respectively). These results indicated that only the intact genome with terminal proteins could be replicated and, in addition, free protein P8 and protein P1 were both required for the replication. This specific replication of PRD1 DNA-genome P8 indicated that elongation proceeded from the P8-dCMP initiation complex and that the nucleotide incorporation was not due to a repair-like activity. As analyzed by alkaline agarose gel electrophoresis (Fig. 8), the length of the replication products increased with time and genome length products were obtained already at 10 minutes.