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Initiation of phage ϕ29 DNA replication *in vitro*: Formation of a covalent complex between the terminal protein, p3, and 5′-dAMP

(protein–dAMP complex/5′-terminal protein/covalent linkage)

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ABSTRACT **Incubation of extracts of ϕ29-infected *Bacillus subtilis* with [α-^{32}P]dATP produced a labeled protein having the electrophoretic mobility of p3, the 5′-terminal protein of ϕ29 DNA. The reaction product was resistant to treatment with micrococcal nuclease, phosphatase, and RNases A and T1 and sensitive to proteinase K. Incubation of the ^{32}P-labeled protein with piperidine under conditions in which the ϕ29 DNA–protein p3 linkage is hydrolyzed released 5′-dAMP. The reaction with [α-^{32}P]dATP was strongly inhibited by anti-p3 serum and required the presence of ϕ29 DNA–protein p3 complex; no reaction took place with proteinase K-treated ϕ29 DNA. These results, together with those of acid hydrolysis and partial proteolysis, indicated that a covalent complex between protein p3 and 5′-dAMP is formed *in vitro*. The initiation complex (protein p3–dAMP) formed in the presence of 0.5 μM [α-^{32}P]dATP can be elongated by addition of 40 μM dNTPs. Treatment with piperidine of the product elongated in the presence of 2′,3′-dideoxycytidine 5′-triphosphate released the expected oligonucleotides, 9 and 12 bases long, taking into account the sequence at the left and right DNA ends, respectively.**

DNA polymerases cannot start new polynucleotide chains because they need a free 3′-OH group for polymerization. Therefore, a key feature in replication is how a DNA chain is initiated. The RNA-priming mechanism will not solve the problem of initiation in the case of a linear DNA since filling the gap at the 5′ ends after RNA excision cannot take place as no 3′-OH end is present. Therefore, a mechanism different from RNA priming must be found to initiate DNA synthesis at the ends of a linear DNA molecule. The existence of a specific protein covalently linked to the 5′ ends of viral DNA molecules, such as those of phage ϕ29 (1–4) and adenovirus (5, 6), has been implicated in solving this problem.

The *Bacillus subtilis* phage ϕ29 genome is a linear double-stranded DNA containing about 18,000 base pairs (7), with a protein, p3, covalently linked to the 5′ ends through a phosphoester bond between the OH group of a serine residue and 5′-dAMP (8). An inverted terminal repetition six nucleotides long is present at the ends of ϕ29 DNA (9, 10). *In vivo* shift-up experiments using temperature-sensitive mutants in cistron 3 have shown that protein p3 is involved in the initiation of DNA replication (11). The replication of ϕ29 DNA starts at both DNA ends, not simultaneously, and occurs by a mechanism of strand displacement (12–14).

A model for the initiation of adenovirus (5) and ϕ29 DNA replication (11–13) has been proposed in which a free molecule of the terminal protein, after recognizing the parental protein at the ends of the DNA, the inverted terminal repetition, or both, reacts with the 5′-terminal dNTP and forms a protein–dNMP covalent linkage, thus providing the 3′-OH group needed by the DNA polymerase. Consistent with this model is the presence of terminal protein linked to the ends of the parental and daughter DNA strands of replicating molecules of adenovirus (15–18) and phage ϕ29 (14). Moreover, a covalent complex between the precursor of the adenovirus terminal protein and 5′-dCMP, the terminal nucleotide at both DNA ends, has been shown recently to be formed *in vitro* (refs. 19–22; P. C. Van der Vliet, personal communication).

In this paper, we show that extracts from ϕ29-infected *B. subtilis* incubated with dATP catalyze the formation of a covalent complex between protein p3 and 5′-dAMP, the 5′-terminal nucleotide at both DNA ends (8–10). Formation of the protein p3–dAMP covalent linkage is specifically inhibited by anti-p3 serum and requires the presence of the ϕ29 DNA–protein p3 complex and ATP; no reaction takes place with proteinase K-treated ϕ29 DNA. Moreover, the complex between protein p3 and dAMP can be elongated to a protein p3-oligonucleotide complex 9 and 12 bases long when 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP) is used, as expected from the sequence at the DNA ends (9, 10). These results support the model in which the terminal protein functions as a primer for the initiation of ϕ29 DNA replication.

MATERIALS AND METHODS

Materials. [α-^{32}P]dATP (410 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), [α-^{32}P]dTTP (410 Ci/mmol), [α-^{32}P]ATP (410 Ci/mmol), and cordycepin [α-^{32}P]5′-triphosphate (3,000 Ci/mmol) were from Amersham. 2′,3′-Dideoxyguanosine 5′-triphosphate (ddGTP) and ddCTP were from P-L Biochemicals. Micrococcal nuclease and RNases A and T1 were from Worthington; alkaline phosphatase from calf intestine was from Boehringer Mannheim; and fungal proteinase K, chromatographically purified, was from Merck. ϕ29 DNA-protein p3 complex was obtained by treatment of phage with 4 M guanidinium chloride for 1 hr at 0°C. The mixture was layered on top of a linear CsCl gradient as described (1), except that centrifugation was for 5.5 hr at 58,000 rpm in an SW 65 rotor. ϕ29 DNA was obtained by phenol extraction from proteinase K-treated phage particles (23). ^{35}S-Labeled protein p3-DNA complex was prepared by J. M. Lázaro and rabbit anti-p3 serum was prepared by I. Prieto.

Growth of Cells and Preparation of Extracts. *B. subtilis* F25-1 or HA101(59)F, lacking DNA polymerase I activity (24), kindly provided by N. Cozzarelli, was grown at 37°C in LB broth up to a cell density of 1×10^8 bacteria per ml and infected with wild-type phage ϕ29 at a multiplicity of 10. At 45 min postinfection, the cells were harvested and washed once with 50 mM

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Abbreviations: ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate.

Tris·HCl, pH 7.5/10% sucrose and then stored at −80°C. Control uninfected cells were processed as the infected cells.

Frozen cells were thawed, suspended in 1/200th the original volume of 50 mM Tris·HCl, pH 7.5/125 mM NaCl containing lysozyme at 500 μg/ml and incubated for 1 hr at 0°C to lyse the cells. Bacterial debris and DNA, both membrane-bound and of high molecular weight, were removed by centrifugation for 30 min at 40,000 × g. The pellet was processed as described below. The supernatant (fraction I), containing 7–10 mg of protein/ml, was treated with 0.47 g of ammonium sulfate per ml. The precipitate was dissolved in a small volume of 50 mM Tris·HCl, pH 7.5/0.1 mM dithiothreitol/20% (vol/vol) glycerol (buffer A) to a protein concentration of 15–25 mg/ml, and the solution was dialyzed against the same buffer (fraction II). Fractions I and II contained endogenous DNA.

The pellet from 2 ml of lysate obtained as indicated above was suspended in 1 ml of 50 mM Tris·HCl (pH 7.5). The bulk of the DNA was removed by phage partition with polyethylene glycol 8000-dextran 500 in 4 M NaCl (25). After a short dialysis to eliminate the salt, polyethylene glycol was removed by addition of ammonium sulfate (0.2 g/ml) and the protein was precipitated from the aqueous phase by addition of ammonium sulfate (0.26 g/ml). The precipitate was dissolved in a small volume of buffer A to a concentration of protein of 7–10 mg/ml, and this solution was dialyzed against the same buffer (fraction III). This fraction also contained endogenous DNA.

To remove nucleic acids, fraction I (≈20 mg of protein) was adjusted to 0.3 M KCl and applied to a DEAE-cellulose column (6 × 1.7 cm) equilibrated with 50 mM Tris·HCl, pH 7.5/0.3 M KCl. The flow-through material was concentrated by addition of ammonium sulfate (0.47 g/ml), the precipitate was dissolved in a small volume of buffer A to a concentration of protein of 15–20 mg/ml, and this solution was dialyzed against the same buffer (fraction IV). All fractions were stored at −80°C for several months with no detectable loss of activity when assayed for formation of the protein p3–dAMP complex in the standard initiation reaction.

Assay for Formation of Protein–dAMP Complex (Initiation Reaction). The standard incubation mixture for the initiation reaction was (final volume, 0.1 ml) 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/3 mM ATP/0.2 μM [α-^{32}P]dATP (10 μCi)/10 μM ddGTP containing ≈200 μg of protein from fraction I, II, or IV or 35 μg of protein from fraction III. When fraction IV was used, 0.7 μg of φ29 DNA-protein p3 complex or proteinase K-treated φ29 DNA was added as indicated. After incubation for 20 min at 30°C or 37°C as indicated, 10 mM EDTA/0.1% $NaDodSO_4$ was added, and the sample was heated for 10 min at 68°C. Free [α-^{32}P]dATP was removed by gel filtration on a Sephadex G-50 column equilibrated with 50 mM Tris·HCl (pH 8.8). Radioactive material eluting in the void volume was treated, unless otherwise indicated, with micrococcal nuclease (250 units/ml) in the presence of 10 mM $CaCl_2$ for 30 min at 37°C, precipitated with 10% trichloroacetic acid, and subjected to electrophoresis in polyacrylamide slab gels containing 10% acrylamide/0.1% $NaDodSO_4$ as described (26). The gels were dried and autoradiography was carried out with Ilford fast tungstate intensifying screens at −80°C. ^{35}S-Labeled φ29 structural proteins were used as molecular weight markers (27).

RESULTS

Formation of Protein p3–dAMP Covalent Linkage. When extracts of φ29-infected *B. subtilis* (fraction I) containing endogenous φ29 DNA (28) were incubated with [α-^{32}P]dATP as described above and the product was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, a ^{32}P-labeled band running in the position of protein p3 was found (Fig. 1*A*, lanes a and c).

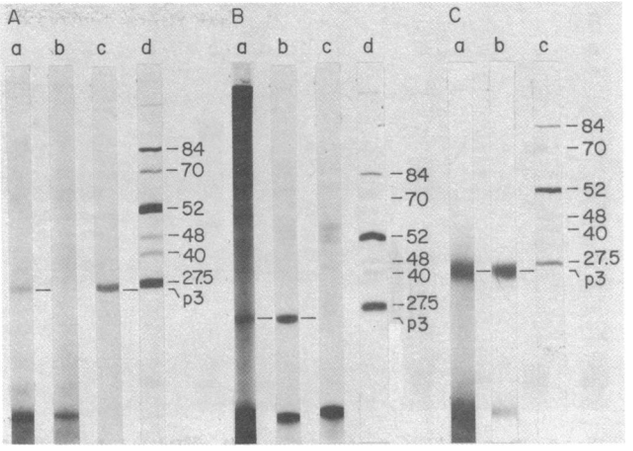

FIG. 1. Labeling of protein p3 by incubation of extracts from φ29-infected cells with [α-^{32}P]dATP. (*A*) Fifty-microliter samples of fraction I from infected (lane a) or uninfected (lane b) cells were incubated for 20 min at 37°C with [α-^{32}P]dATP in the presence of ddGTP, processed, and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Lane c: ^{35}S-labeled protein p3 prepared by treatment of ^{35}S-labeled protein p3–DNA complex with micrococcal nuclease to degrade the DNA up to the last nucleotide (8). (*B*) Ten-microliter samples of fraction II from infected (lanes a and b) or uninfected (lane c) cells were incubated for 20 min at 37°C and processed as described in *Materials and Methods* except that the sample in lane a was not treated with micrococcal nuclease. (*C*) Ten-microliter samples of fraction III from infected cells were incubated for 20 min at 30°C and processed without (lane a) or with (lane b) micrococcal nuclease treatment. M_r markers: *A*, lane d; *B*, lane d; C, lane c; values given are $\times 10^{-3}$.

No ^{32}P-labeled band having this electrophoretic mobility was produced when extracts from uninfected cells were used (Fig. 1*A*, lane b). To increase the efficiency of labeling, the extracts were treated with ammonium sulfate to eliminate endogenous nucleotides (fraction II). After incubation with [α-^{32}P]dATP, analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis showed the presence of an abundant ^{32}P-labeled band running in the position of protein p3 (Fig. 1*B*). The position of the band was the same whether or not the sample was treated with micrococcal nuclease before electrophoresis (Fig. 1*B*, lanes a and b), although the amount of ^{32}P-labeled material running down the gel was drastically reduced by this treatment. The amount of radioactivity (^{32}P) remaining in the protein p3 band after nuclease treatment was 3.5 fmol. When fraction II from extracts of uninfected cells was incubated with [α-^{32}P]dATP, no radioactive band in the position of protein p3 was found (Fig. 1*B*, lane c). Fig. 1*C* shows the formation of the ^{32}P-labeled protein p3 band when fraction III was used without (lane a) or with (lane b) micrococcal nuclease treatment. The amount of ^{32}P radioactivity in this band was 3.3 fmol. The amount of ^{32}P-labeled material running down the gel in the sample untreated with micrococcal nuclease is much less with fraction III than with fraction II.

The ^{32}P-labeled protein p3 band was drastically decreased when ATP was omitted (Fig. 2*A*, lanes a and b) and disappeared completely in the absence of $MgCl_2$ (lane c) or in the presence of 0.35 M KCl (lane d), a concentration that does not inhibit φ29 DNA elongation in the *in vitro* system (28). When [α-^{32}P]dTTP, [α-^{32}P]ATP, or [α-^{32}P] cordycepin 5′-triphosphate was substituted for [α-^{32}P]dATP, no ^{32}P-labeled band at the position of protein p3 was detected (Fig. 2*B*, lanes a–c). The labeled bands present when the incubation was carried out in the absence of ATP (Fig. 2*A*, lane b) or when [α-^{32}P]ATP was used instead of [α-^{32}P]dATP (Fig. 2*B*, lane b) are sensitive to proteinase K treat-

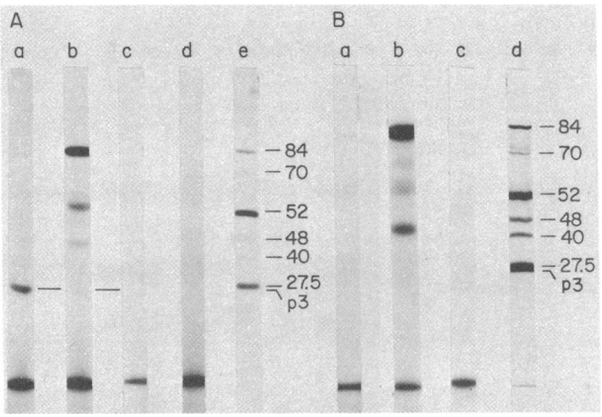

FIG. 2. Requirements for formation of the ^{32}P-labeled protein p3 band. (*A*) Ten-microliter samples of fraction II from infected cells were incubated for 20 min at 37°C with [α-^{32}P]dATP, ATP as indicated, and ddGTP, processed, and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Lanes: a, control; b, without ATP; c, without $MgCl_2$; d, with 0.35 M KCl. (*B*) As in *A* except that [α-^{32}P]dATP was absent. Lanes: a, with [α-^{32}P]dTTP (10 μCi); b, with [α-^{32}P]ATP (10 μCi; 0.24 μM); c, with cordycepin 5′-[α-^{32}P]triphosphate (10 μCi). M_r markers: *A*, lane e; *B*, lane d; values given are $\times 10^{-3}$.

ment, and they were present when extracts from uninfected cells were used.

The ^{32}P-labeled protein p3 band remained after treatment with phosphatase or RNase A and RNase T1, although most of the other ^{32}P-labeled bands disappeared after phosphatase treatment (Fig. 3, lanes a–c). Most of the bands present when extracts from uninfected cells were used also disappeared after phosphatase treatment (lanes f and g). By treatment with either proteinase K or piperidine (under conditions in which the protein p3–DNA linkage is hydrolyzed; unpublished results), the ^{32}P-labeled protein p3 band completely disappeared (lanes d and e). Lane h shows the electrophoretic mobility of ^{35}S-labeled protein p3 released from the DNA–p3 complex by treatment with piperidine.

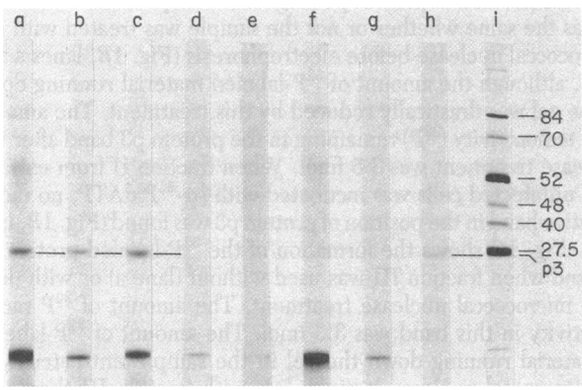

FIG. 3. Sensitivity of the ^{32}P-labeled protein p3 band to various treatments. Ten-microliter samples of fraction II from infected (lanes a–e) or uninfected (lanes f and g) cells were incubated for 20 min at 37°C with [α-^{32}P]dATP and ddGTP, treated with micrococcal nuclease as indicated below, and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Lanes: a, control; b, with phosphatase (1 unit/ml) for 30 min at 37°C; c, with RNase A (5 μg/ml) and RNase T1 (4 units/ml) for 30 min at 37°C; d, with proteinase K (200 μg/ml) in the presence of 0.1% NaDodSO$_4$ for 3 hr at 37°C; e, with 0.5 M piperidine for 2 hr at 37°C; f, uninfected control; g, uninfected, treated with phosphatase as for lane b; h, ^{35}S-labeled protein p3 prepared from ^{35}S-labeled protein p3–DNA complex by treatment with micrococcal nuclease and then with 0.5 M piperidine for 2 hr at 37°C; i, M_r markers (values are $\times 10^{-3}$).

Characterization of the Protein Labeled with [α-^{32}P]dATP as p3. To determine whether the protein labeled *in vitro* with [α-^{32}P]dATP was p3, the effect of anti-p3 serum on the reaction was tested. Fraction III from infected cells was incubated with serum against protein p3 before addition of the remaining components of the *in vitro* initiation reaction. The labeling of the protein p3 band was strongly inhibited (≈65%; Fig. 4, lanes d and e) compared with experiments in which no serum (lane a) or nonimmune serum (lanes b and c) were added. Additional indications that the protein labeled *in vitro* was p3 were that (*i*) acid hydrolysis of the ^{32}P-labeled protein yielded phosphoserine under conditions in which this product was formed from the ϕ29 DNA–protein p3 complex (8) and (*ii*) partial hydrolysis with V8 protease (29) of the ^{32}P-labeled protein and ^{35}S-labeled protein p3 produced a common peptide having the same electrophoretic mobility in NaDodSO$_4$/polyacrylamide gels (data not shown).

Characterization of the Nucleotide Moiety Covalently Linked to Protein p3. Since the sequence at both 5′ ends of ϕ29 DNA is A-A-A-G-T-A (9, 10), the reaction of protein p3 with [α-^{32}P]dATP in the presence of ddGTP would stop at the fourth nucleotide if elongation takes place *in vitro* or at the first adenosine if no elongation occurs. It is known that, after digestion of the protein p3–DNA complex with micrococcal nuclease, pdAp remains attached to the protein (8). The ^{32}P-labeled protein p3 formed in the standard incubation of fraction III with [α-^{32}P]dATP and ddGTP, either with or without micrococcal nuclease treatment, was eluted from a polyacrylamide gel. In both cases, treatment with 0.5 M piperidine for 2 hr at 37°C, conditions that hydrolyze the protein p3–DNA linkage, released a radioactive product that comigrated with 5′-dAMP on TLC on polyethyleneimine cellulose (Fig. 5, lanes b–d). No radioactivity at the position of 5′-dAMP appeared when the sample was not treated with piperidine (lane a). The fact that the sample that had not been treated with micrococcal nuclease released 5′-dAMP as the major product (lane b) indicates that, in the presence of 0.2 μM dATP, most of the protein p3–dAMP complex is not elongated further. This conclusion is supported by the fact that, in the sample that had been treated with micrococcal nuclease, only a small amount of the radioactivity remained close to the origin, presumably as pdAp, since it ran in the position of ADP and treatment with phosphatase released its radioactivity at the P_i position (lane d). The radioactivity that remains at the origin after piperidine treatment in the sample

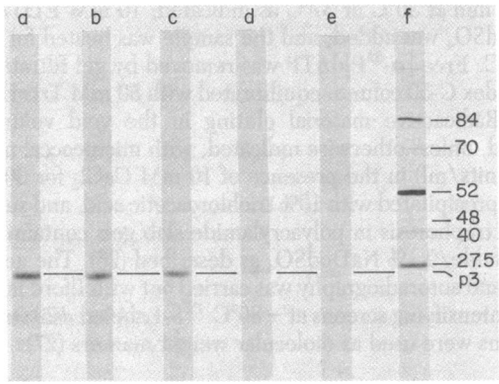

FIG. 4. Inhibition by anti-p3 serum of formation of the ^{32}P-labeled protein p3 band. Five-microliter samples of fraction III from infected cells were incubated for 10 min (lanes b and d) or 30 min (lanes c and e) with 5 μl of nonimmune serum (lanes b and c) or anti-p3 serum (lanes d and e) and then for 20 min at 30°C with [α-^{32}P]dATP and ddGTP, processed, and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Lane f, M_r markers (values are $\times 10^{-3}$).

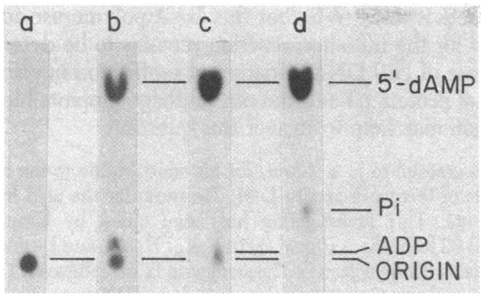

FIG. 5. Characterization of the nucleotide moiety linked to the ^{32}P-labeled protein p3 band. The ^{32}P-labeled protein p3 from untreated samples (lanes a and b) or samples treated (lanes c and d) with micrococcal nuclease was eluted from the gel by overnight incubation with 50 mM Tris·HCl, pH 7.5/0.1% NaDodSO$_4$. The protein was precipitated with acetone and suspended in water. A sample from each was applied to a polyethyleneimine-cellulose plate before (lane a) or after (lanes b–d) incubation with 0.5 M piperidine for 2 hr at 37°C followed by removal of the piperidine. The sample in lane d was treated with phosphatase (6 units/ml) for 30 min at 37°C before treatment with piperidine. In all cases, 5'-dAMP was added as an internal marker. 5'-ADP and P$_i$ were also run as markers. The chromatogram was developed first in 0.5% formic acid and then in 0.15 M lithium formate (pH 3.0) in the same dimension.

not treated with the nuclease (lane b) is probably due to DNA contamination in the region of the gel corresponding to the protein p3 band (see Fig. 1*C*).

Elongation of the Protein p3–dAMP Complex. To test whether or not the protein p3–dAMP complex formed *in vitro* can serve as a primer for elongation, a pulse–chase experiment was carried out. Fraction III from infected cells was incubated with 0.5 μM [α-^{32}P]dATP for 5 min (pulse). Then, 40 μM dATP/40 μM dGTP/40 μM dTTP/40 μM dCTP was added and incubation was continued up to 20 min (chase). The samples were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and the ^{32}P radioactivity in the protein p3 band was quantitated by densitometry. About 40% of the ^{32}P radioactivity in the protein p3 band at the pulse (Fig. 6*A*, lane a) was chased into high molecular weight material (lane b). Since the nucleotide sequence at the left and right ϕ29 DNA 5′ ends is A-A-A-G-T-A-A-G-C . . . and A-A-A-G-T-A-G-G-G-T-A-C . . ., respectively (9, 10), 100 μM ddCTP (instead of dCTP) was added to a sample during the chase to stop the elongation at nucleotides 9 and 12. Under these conditions, about 40% of the radioactivity appeared in a new band having an electrophoretic mobility less than that of the protein p3–dAMP band, corresponding to a molecular weight of 31,000 (Fig. 6*A*, lane c). When the M_r 31,000 band was eluted from the gel, hydrolyzed with piperidine, and analyzed in a denaturing polyacrylamide gel, bands corresponding to oligonucleotides 9 and 12 bases long were seen (Fig. 6*B*, lane a). This is the expected result for products elongated in the presence of ddCTP. In addition, bands running in the positions of the di- and pentanucleotide were present. The band moving at the dinucleotide position was shown to be 5′-dAMP, probably due to contamination with the material from the main band. The pentanucleotide band could represent a premature stop in elongation. No bands at any of the above positions appeared when the piperidine treatment was omitted (lane b).

In an experiment in which the dATP concentration was maintained at 0.5 μM during the entire incubation period in the presence of 40 μM dGTP/40 μM dTTP/100 μM ddCTP, the amount of the M_r 31,000 band was much smaller than when 40 μM dATP was added after 5 min. When the dATP concentration was maintained at 40 μM during the entire incubation period, about 2/3 of the radioactivity was present in the M_r 31,000 band

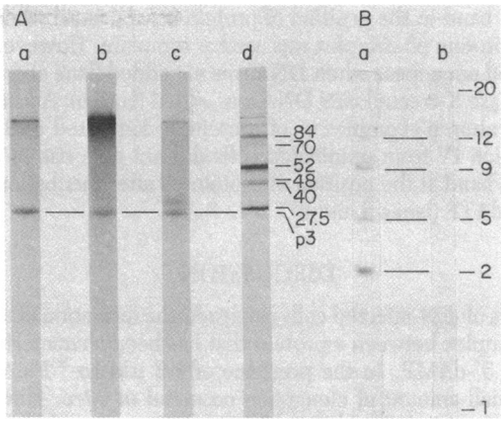

FIG. 6. Elongation of the protein p3–dAMP complex. (*A*) Thirty-microliter samples of fraction III from infected cells were incubated in a volume of 0.3 ml in the presence of 0.5 μM [α-^{32}P]dATP (200 Ci/mmol). After 5 min at 30°C, a 0.1-ml sample was removed and the reaction was stopped (lane a); 40 μM dATP/40 μM dGTP/40 μM dTTP/40 μM dCTP (lane b) or 40 μM dATP/40 μM dGTP/40 μM dTTP/100 μM ddCTP (lane c) was added to samples of 0.1 ml and incubation was continued for an additional 15 min. The samples were processed as described in *Materials and Methods* except micrococcal nuclease treatment was not carried out and electrophoresis was in NaDodSO$_4$/20% acrylamide slab gels. Lane d, M_r markers (values given are $\times 10^{-3}$). (*B*) The ^{32}P-labeled band with an apparent M_r of 31,000 was eluted from the gel by overnight incubation with 50 mM Tris·HCl, pH 7.5/0.1% NaDodSO$_4$, precipitated with acetone and suspended in water. Lanes: a, half of the sample was treated with 0.5 M piperidine for 2 hr at 37°C and, after removal of the piperidine, subjected to electrophoresis in a 20% polyacrylamide denaturing gel as described (10); b, the other half was not treated with piperidine but was subjected to electrophoresis as a control. The reaction products from a chemical sequence analysis of DNA fragments labeled with T4 polynucleotide kinase were run in parallel lanes as markers (gift from C. Escarmís).

(results not shown). These findings suggest that a high concentration of dATP is required to elongate the initiation complex.

Template Requirement for Formation of the Protein p3–dAMP Covalent Linkage. The endogenous ϕ29 DNA was removed from the extracts by DEAE-cellulose chromatography (fraction IV), and this fraction was used as a protein source for the reaction with [α-^{32}P]dATP. Fig. 7*A* (lane c) shows that a ^{32}P-

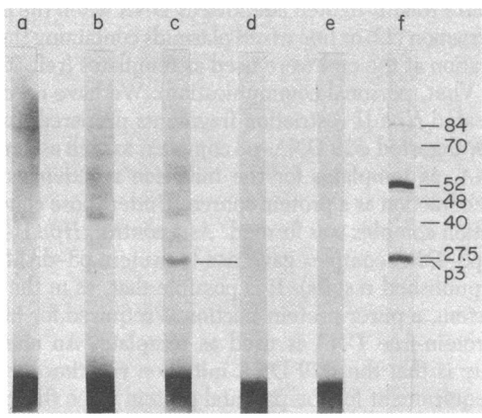

FIG. 7. Template requirement for formation of the protein p3–dAMP complex. Ten-microliter samples of fraction IV from infected (lanes a–c) or uninfected (lanes d and e) extracts were incubated for 20 min at 30°C with [α-^{32}P]dATP and ddGTP and no other additions (lane a) or with 0.7 μg of proteinase K-treated ϕ29 DNA (lanes b and d) or 0.7 μg of ϕ29 DNA–protein p3 complex (lanes c and e), processed, and subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis. Lane f: M_r markers (values given are $\times 10^{-3}$).

labeled band in the position of protein p3 appeared when ϕ29 DNA–protein p3 complex was used as template. However, this band did not appear when DNA was not added (lane a) or when proteinase K-treated ϕ29 DNA was added (lane b). Addition of ϕ29–protein p3 complex or of proteinase K-treated ϕ29 DNA to fraction IV from uninfected cells did not give rise to a ^{32}P-labeled band at the position of protein p3 after incubation with [α-^{32}P]dATP (lanes d and e).

DISCUSSION

Extracts of ϕ29-infected cells catalyzed the formation of a covalent complex between a protein that has been characterized as p3 and 5'-dAMP. In the presence of 0.2 μM [α-^{32}P]dATP, a very small amount of elongation occurred *in vitro*. However, when 40 μM dATP/dGTP/dTTP/dCTP was added after a pulse, about 40% of the p3–dAMP complex was chased into high molecular weight material. Moreover, when 100 μM ddCTP was added instead of dCTP, to stop the elongation reaction at nucleotides 9 and 12, according to the sequence at the left and right DNA ends, respectively, a labeled M_r 31,000 band that released oligonucleotides 9 and 12 bases long after piperidine treatment was formed. The results suggest that the elongation reaction requires a high nucleotide concentration since, when a low dATP concentration (0.2–0.5 μM) was maintained during the incubation, a very small amount of elongated product was obtained, consistent with the finding of protein p3–dAMP as the major product. The fact that a complete chase of the protein p3–dAMP complex was not obtained under those conditions suggests that, for efficient elongation, this reaction must be coupled to initiation. In support of this idea is the fact that, when 40 μM dATP was used during the entire incubation period, about 2/3 of the product was in an elongated form.

When endogenous DNA was eliminated from extracts of ϕ29-infected cells, formation of the initiation complex was dependent on the addition of protein p3–DNA complex as template. No reaction took place with proteinase K-treated ϕ29 DNA. A similar result has been obtained with the adenovirus system (19–22). However, it has been reported recently that, when the residual peptides remaining after Pronase treatment of adenovirus DNA were eliminated by piperidine treatment, protein-free DNA restriction fragments supported the initiation reaction when a partially purified protein fraction was used but not with crude extracts (22). The initiation reaction also occurred with denatured Pronase-treated adenovirus DNA when the purified protein fraction (22) or linearized plasmids containing the origin of replication at the end were used as templates (ref. 22; P. C. Van der Vliet, personal communication). We have used piperidine-treated *Hpa* II restriction fragments prepared from proteinase K-digested ϕ29 DNA–p3 complex, as well as denatured ϕ29 DNA, as templates for the initiation reaction using the DNA-free fraction as a protein source. Under those conditions, no initiation complex was formed. As a control, *Hpa* II-treated protein p3–DNA complex gave rise to protein p3–dAMP complex (unpublished results). It is possible that, as in the adenovirus system, a purer protein fraction is required for initiation when protein-free DNA is used as template. An alternative possibility is that the ϕ29 DNA initiation reaction has an absolute requirement for the parental protein since the inverted terminal repetition is only 6 nucleotides long (9, 10) in contrast to the adenovirus DNA one of about 100 nucleotides (30–34).

An important question that remains is whether protein p3 itself or some other enzymatic activity, bacterial or phage-encoded, catalyzes the formation of the protein–dAMP complex. The precursor of the terminal protein from adenovirus-infected cells has been purified and shown to copurify with a DNA polymerase activity (35). Whether this DNA polymerase activity is required for the initiation reaction remains to be determined. In the case of ϕ29 DNA initiation of replication *in vitro*, purification of protein p3 and the use of phage mutants blocked in replication may help to answer this question.

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