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Terminal protein-primed DNA amplification

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ABSTRACT **By using appropriate amounts of four bacteriophage ϕ29 DNA replication proteins—terminal protein, DNA polymerase, protein p6 (double-stranded DNA-binding protein), and protein p5 (single-stranded DNA-binding protein)—it has been possible to amplify limited amounts of the 19,285-bp-long ϕ29 DNA molecule by three orders of magnitude after 1 hr of incubation at 30°C. Moreover, the quality of the amplified material was demonstrated by transfection experiments, in which infectivity of the synthetic (amplified) ϕ29 DNA, measured as the ability to produce phage particles, was identical to that of the natural ϕ29 DNA obtained from virions. The results presented in this paper establish some of the requisites for the development of isothermal DNA amplification strategies based on the bacteriophage ϕ29 DNA replication machinery that are suitable for the amplification of very large (>70 kb) segments of DNA.**

The genome of *Bacillus subtilis* phage ϕ29 consists of a linear double-stranded DNA (19,285 bp), with a 6-bp-long inverted terminal repeat and a terminal protein (TP) covalently linked at both 5′ ends (reviewed in ref. 1). Since the first proposal by Rekosh *et al.* (2), by which a free molecule of TP would act as a primer to initiate synthesis at both ends of the linear DNA molecule, the availability of *in vitro* replication systems for both adenovirus and bacteriophage ϕ29 allowed the confirmation of this hypothesis and the characterization of the functional role of other replication proteins involved in this process (reviewed in ref. 1).

As depicted in Fig. 1, initiation of ϕ29 DNA replication is triggered by the viral protein p6 (double-stranded DNA-binding protein; DBP), which forms a nucleoprotein complex at both ϕ29 DNA ends, producing a conformational change in the DNA that probably leads to local opening of the DNA duplex (5). The primer TP forms a 1:1 complex with ϕ29 DNA polymerase that recognizes both ends of the linear ϕ29 DNA molecule (replication origins). Then, in the presence of dATP and Mg^{2+}, ϕ29 DNA polymerase catalyzes the formation of a covalent bond between dAMP and the OH group of Ser-232 of the TP acting as primer (6, 7). In this reaction, dATP is selected by base complementarity with the second 3′-nucleotide of the template strand (8). After this initiation step, dissociation of the TP–DNA polymerase heterodimer is likely to occur (transition) to replace the TP–DNA polymerase interactions required for initiation by the DNA polymerase–DNA interactions required for the elongation of the newly created DNA primer. Concomitantly, an asymmetric translocation (sliding back) of only TP-dAMP, but not of the template, followed by addition of a new dAMP residue, allows the recovery of the information corresponding to the first template nucleotide (8). During elongation, ϕ29 DNA polymerase catalyzes highly processive polymerization coupled to strand displacement (9), and, therefore, complete replication of both strands proceeds continuously from each terminal priming event. As the two replication forks move, DNA synthesis is initially coupled to strand displacement of long stretches of single-stranded ϕ29 DNA, producing type I replicative intermediates (see Fig. 1). When the two replication forks, moving in opposite directions, merge, a new type of replicative intermediate (type II) is formed. Electron microscopy analysis of ϕ29 replicative intermediates *in vitro* showed that the viral protein p5 binds to the single-stranded portion of both type I and II molecules, thus acting as a single-stranded DNA-binding protein (SSB) during ϕ29 DNA replication (4). Once polymerization of both strands has been completed, the two DNA polymerase molecules dissociate from the DNA to reassume initiation and replication of a new ϕ29 DNA molecule.

The symmetrical mode of ϕ29 DNA replication is similar in several aspects (primed initiation at the ends of a linear DNA molecule and continuous synthesis of both strands) to the most widely used DNA amplification technique: PCR. However, the natural ϕ29 DNA amplification system has significant differences derived from the nature of the primers (TP), from the fact that both DNA ends are true replication origins and, therefore, no thermal denaturation is required to position primers; and from the specific strand displacement coupled to DNA synthesis catalyzed by ϕ29 DNA polymerase, which allows this enzyme to replicate extremely long double-stranded DNA molecules in isothermal conditions.

In this paper, as the initial step in the development of amplification vectors based on the ϕ29 DNA replication machinery, we have characterized the minimal protein factors required *in vitro* for the efficient TP-primed amplification of limited amounts of ϕ29 DNA.

MATERIALS AND METHODS

Nucleotides, Proteins, and DNA Templates. Unlabeled dNTPs and [α-^{32}P]dNTPs (410 Ci/mmol; 1 Ci = 37 GBq) were obtained from Amersham. ϕ29 DNA polymerase (M_r = 66,520) and TP (M_r = 30,918) were overproduced in *Escherichia coli* cells and purified essentially as described (10, 11). Protein p6 (M_r = 11,873) and protein p5 (M_r = 13,212), obtained from ϕ29-infected *B. subtilis* cells, were purified as described (12, 13). TP-linked DNA from ϕ29 *sus14* (1242) virions, isolated as described (14), was used as input template for *in vitro* amplification experiments and as a control DNA in transfection experiments.

Phage and Bacterial Strains. Bacteriophage ϕ29 *sus14* (1242) has a nonsense (*sus*) mutation in gene *14* (15), which produces a delayed lysis phenotype when plated in the nonpermissive strain *B. subtilis* 110NA Try$^-$ *spoA*$^-$ (16). *B. subtilis* BG295 (Sup 3) (17) was used for transfection experiments. *B. subtilis* MO-101-P *spoA*$^-$ Thr$^-$ (Met$^-$)$^+$ Su$^+$44 (18)

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Abbreviations: TP, terminal protein; pfu, plaque-forming unit(s); SSB, single-stranded DNA-binding protein (p5); DBP, double-stranded DNA-binding protein (p6).

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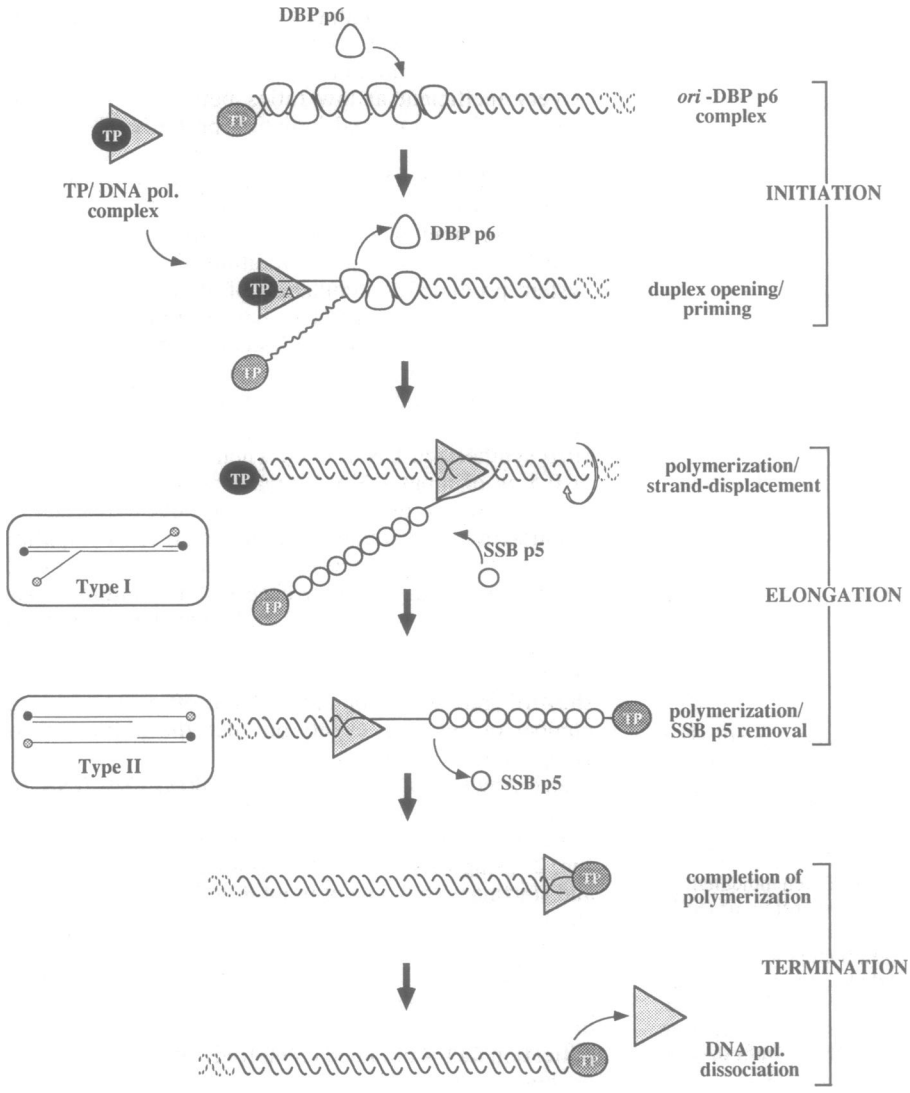

FIG. 1. Different stages and viral gene products involved in ϕ29 DNA replication. For simplicity, only one ϕ29 DNA end (replication origin) is represented. ϕ29 TP is indicated in black (when acting as primer) or shadowed (parental). ϕ29 DNA polymerase is depicted by a triangle. The two main types of replicative intermediates (I and II) produced during the elongation stage and observed both during *in vivo* (3) and *in vitro* (4) ϕ29 DNA replication are represented in boxes.

was used as permissive host (suppressor strain) for titration of ϕ29 *sus14* (1242).

ϕ29 DNA Amplification Assay. To optimize the initiation step of ϕ29 DNA replication, an equimolar (1:1) complex between highly purified ϕ29 DNA polymerase and TP was obtained by incubation of both proteins in the presence of 20 mM ammonium sulfate, as described (19). The incubation mixture contained, in 10 μl, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 4% glycerol, bovine serum albumin (0.1 mg/ml), 80 μM each dCTP, dGTP, dTTP, and [α-^{32}P]dATP (2 μCi), 15 ng of preformed TP–DNA polymerase complex, and 20 ng of free TP. As indicated, different amounts of ϕ29 DNA (containing parental TP), obtained from phage ϕ29 mutant *sus14* (1242), were used as input DNA template. When indicated, different amounts of purified proteins p6 (DBP) and p5 (SSB) were either individually or simultaneously added. After incubation for 1 hr at 30°C, the reaction was stopped by addition of 10 mM EDTA, and the unreacted [α-^{32}P]dATP was removed by filtration through a Sephadex G-50 spin column in the presence of 0.1% SDS. Quantitation of the DNA synthesized *in vitro*, measured as the total amount (in nanograms) of dNTP incorporated, was carried out from the amount of radioactivity (Cerenkov radiation) corresponding to the excluded volume. When indicated, the size of the amplified DNA was analyzed by alkaline agarose gel electrophoresis (20) followed by autoradiography and ethidium bromide staining.

Infectivity Assay for the *in Vitro*-Amplified ϕ29 DNA. In this case, the *in vitro* amplification conditions were as described above except that the input ϕ29 DNA (0.5 ng) was incubated with TP–DNA polymerase complex (15 ng), free TP (20 ng), DBP (10 μg), and SSB (8 μg), in the presence of the four unlabeled dNTPs each at 80 μM. After 2 hr at 30°C, the reaction was stopped with 10 mM EDTA, and the DNA was precipitated with ethanol and resuspended in 10 mM Tris·HCl (pH 7.5)/0.1 mM EDTA. As an *in vivo* control of infectivity, 500 ng of the ϕ29 DNA used as input for amplification was incubated under exactly the same conditions but in the absence of dNTPs. After 2 hr at 30°C, the control ϕ29 DNA was processed as described for the *in vitro*-amplified ϕ29 DNA. Aliquots of the amplified ϕ29 DNA were quantitated by alkaline agarose gel electrophoresis (20) and ethidium bromide staining. Different amounts of ϕ29 DNA (control or amplified) were used to transfect *B. subtilis* BG295 (suppressor strain)-competent cells, prepared as described (21). After transfection, infectivity, expressed as plaque-forming units (pfu), was determined by plating on *B. subtilis* Su^+44 suppressor strain.

RESULTS

Requirements for *in Vitro* Amplification of ϕ29 DNA. It was reported that the complete replication of both ϕ29 DNA strands *in vitro* required only two proteins: the ϕ29 TP, which acts as initiation primer, and the viral DNA polymerase (22).

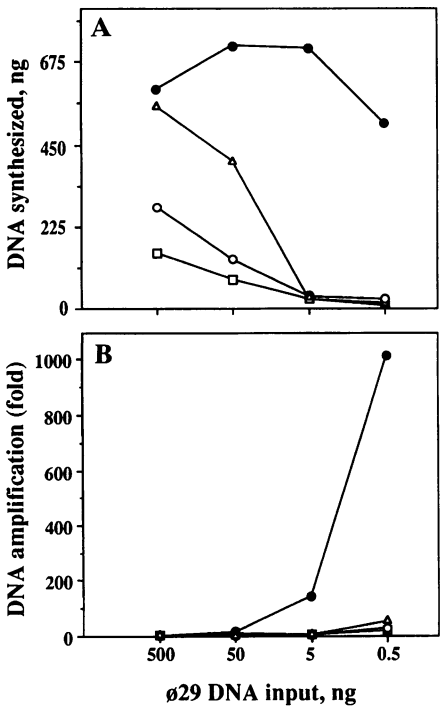

FIG. 2. *In vitro* amplification of ϕ29 DNA. (*A*) DNA synthesized *in vitro* with different combinations of ϕ29 DNA replication proteins, as a function of the amount of input ϕ29 DNA. The ϕ29 DNA amplification assay was carried out as described in *Materials and Methods*, in the presence of 15 ng of preformed TP–DNA polymerase complex, 20 ng of free TP, and different amounts of ϕ29 DNA (containing parental TP) as input DNA template. When indicated, 10 μg of purified protein p6 (DBP) and 8 μg of purified protein p5 (SSB) were added either individually (○ and △) or simultaneously (●) to the minimal system (□) previously described. After incubation for 1 hr at 30°C, the reaction was stopped and quantitated as the total amount (in nanograms) of dNTP incorporated. (*B*) DNA amplification factors, corresponding to the different conditions shown in *A*, were calculated as the ratio between the amount of DNA synthesized *in vitro* and the amount of input ϕ29 DNA. □, DNA polymerase + TP; ○, DNA polymerase + TP + p6; △, DNA polymerase + TP + p5; ●, DNA polymerase + TP + p6 + p5.

However, this minimal system was defined using a considerable amount of ϕ29 DNA template (0.5 μg; 1.6 nM), and, therefore, we reconsidered the characterization of the proteins required for an efficient DNA amplification *in vitro* (i.e., starting from limited amounts of ϕ29 DNA).

Fig. 2*A* shows that the amount of DNA synthesized when using a minimal system formed by TP and DNA polymerase is strongly dependent on the amount of input ϕ29 DNA. Thus, *in vitro* synthesis occurred at input doses of 500 ng (4 nM) and 50 ng (0.4 nM), but it was almost undetectable at input doses corresponding to 0.5 ng (4 pM) and 5 ng (0.04 nM); no reaction was observed even after overnight incubation. Therefore, initiation is probably the rate-limiting step: it is very inefficient at low ratios between DNA replication origins and the minimal initiation proteins (TP–DNA polymerase complex). As expected from previous data (23), addition of DBP to the *in vitro* minimal system produced only a 1.5- to 2-fold increase in the amount of DNA synthesized at any of the input ϕ29 DNA doses tested. Also in agreement with previous reports (24), addition of SSB to the minimal system produced a stimulation of about 4- to 5-fold in the amount of synthesized DNA when starting with either 500 ng or 50 ng of input ϕ29 DNA; however, at the lower doses of input DNA tested, the effect of adding SSB was negligible. On the other hand, the simultaneous addition of viral DBP and SSB to the minimal system resulted in a high yield of *in vitro*-synthesized DNA, even when starting with either 5 ng or 0.5 ng of input DNA template. Analysis of the titration curves shown in Fig. 3 indicates that both DBP and SSB are required in high amounts to obtain an efficient amplification.

Thus, in the presence of the four viral proteins, it was possible to amplify by three orders of magnitude the amount of input ϕ29 DNA (see Fig. 2*B*). Under these conditions, an exponential increase of *in vitro*-synthesized DNA could be obtained as a function of the time of incubation (data not shown), indicating not only a high efficiency in terms of number of initiation events but also the occurrence of full elongation of both DNA strands.

Size Analysis of the *in Vitro*-Amplified ϕ29 DNA. Fig. 4 (lanes 1–4) shows the size analysis of the amplified ϕ29 DNA obtained *in vitro* in an experiment similar to that shown in Fig. 2, using each of the four combinations of viral proteins and starting with 0.5 ng of input ϕ29 DNA. Interestingly, the amplified DNA, obtained only in the presence of the four viral proteins (Fig. 4, lane 4), corresponded to full-length ϕ29 DNA. Moreover, electron microscopy analysis of the DNA amplified under these conditions indicated the accumulation of mature ϕ29 DNA molecules (data not shown). The amplification factor obtained (1460-fold) implies a maximal time of about 6 min per duplication cycle; the amount of ϕ29 DNA synthesized under these conditions (730 ng/58 fmol) is close to the limit imposed by the amount of dNTPs provided.

A delicate equilibrium (stoichiometry) of initiation and elongation factors required for the efficient *in vitro* amplification of ϕ29 DNA can be clearly observed in Fig. 4, lanes 5–9. At a fixed concentration of DBP and SSB, a small increase in the amount of TP–DNA polymerase complex with respect to the one allowing optimal amplification of full-length ϕ29 DNA leads to the generation of immature elongation products, although the amount of DNA synthesized was roughly similar. This result is interpreted as the consequence of an imbalance between the number of initiations (increased) and the ratio of elongation factors to growing

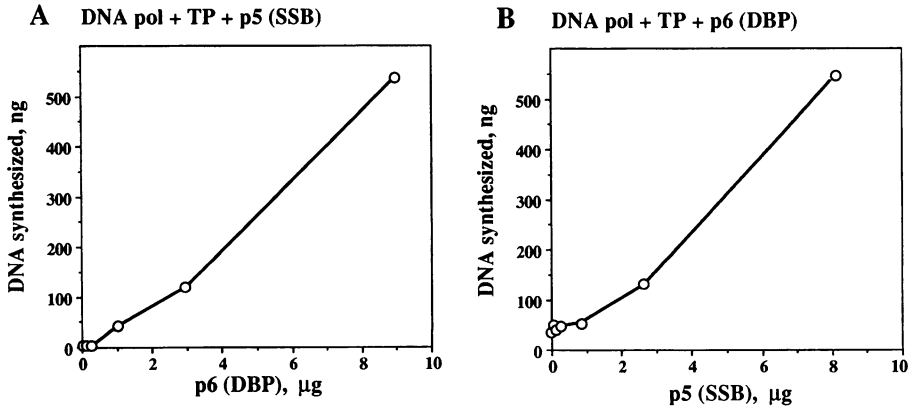

FIG. 3. Requirement of proteins p6 (DBP) and p5 (SSB) for ϕ29 DNA amplification. The ϕ29 DNA amplification assay was carried out as described in *Materials and Methods*, but using 0.5 ng of ϕ29 DNA (containing parental TP) as input DNA template, 15 ng of preformed TP–DNA polymerase complex, 20 ng of free TP, and either 8 μg of SSB and different amounts of DBP (*A*) or 10 μg of protein DBP and different amounts of SSB (*B*). After incubation for 1 hr at 30°C, the reaction was stopped and quantitated as the total amount (in nanograms) of dNTP incorporated.

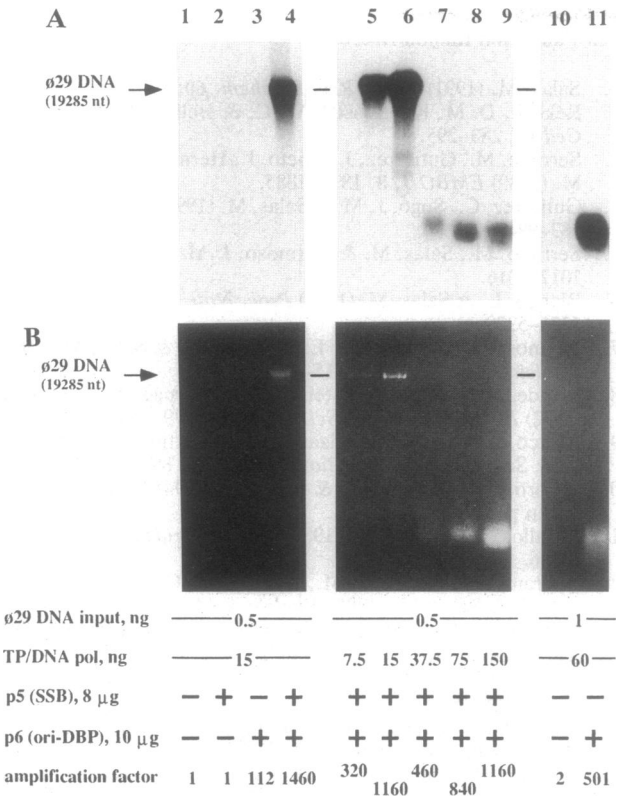

FIG. 4. Size analysis of *in vitro*-amplified ϕ29 DNA. Amplification assays were carried out essentially as described in *Materials and Methods*, using the indicated amounts of input ϕ29 DNA. Different ϕ29 DNA replication proteins [TP–DNA polymerase complex, p6 (DBP), and p5 (SSB)] were added as indicated. After 1 hr of incubation at 30°C, the reaction was stopped with 10 mM EDTA, the amount of synthesized DNA was quantitated as described, and the size of the amplified DNA was analyzed by alkaline agarose gel electrophoresis followed by autoradiography (*A*) and ethidium bromide staining (*B*). The amplification factor corresponding to each particular condition, calculated as described in the legend to Fig. 2, is indicated. The arrows at the left indicate the electrophoretic mobility of full-length ϕ29 DNA obtained from virions.

DNA chains (reduced). Moreover, the size of the synthesized DNA was linearly dependent on the amount of SSB (data not shown). On the other hand, in the absence of SSB, and using a large amount (60 ng) of TP–DNA polymerase complex, it was possible to observe a large stimulation in the amount of short elongation products as a consequence of adding DBP (Fig. 4, lanes 10 and 11). This result allows us to conclude that binding of DBP to the ϕ29 DNA replication origins is the main factor stimulating the initiation stage in these *in vitro* amplification conditions. Therefore, it is tempting to speculate that, in addition to facilitating the opening of ϕ29 DNA replication origins, DBP increases the affinity of the TP–DNA polymerase complex by the ϕ29 DNA ends. On the other hand, SSB, in addition to preventing the nonproductive binding of replication proteins to the displaced strands (25), appears to be critical for the progression (maturation) of the multiple replication forks initiated at both ϕ29 DNA replication origins.

All these results agree with the importance of the four viral proteins for ϕ29 DNA replication *in vivo* (26, 27) and also emphasize the necessity of a precise stoichiometry of these proteins to maintain the equilibrium between the initiation and elongation stages of replication.

Infectivity of the *in Vitro*-Amplified ϕ29 DNA. As a quality control of the *in vitro* amplification procedure described in this paper, the infectivity of the synthetic (*in vitro*) ϕ29 DNA,

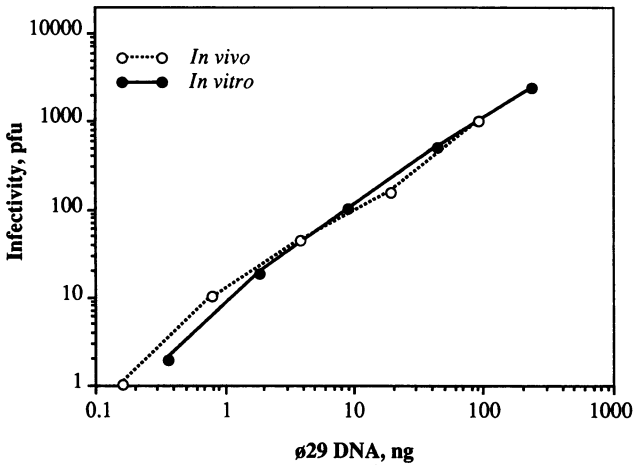

FIG. 5. Infectivity of the *in vitro*-amplified ϕ29 DNA. *In vitro* amplification conditions were as described in *Materials and Methods* for the infectivity assay. A control ϕ29 DNA (*in vivo*) was processed essentially in the same form but in the absence of dNTPs. The indicated amounts of either *in vivo* (control) or *in vitro* ϕ29 DNA were used to transfect *B. subtilis* BG295 (suppressor strain) competent cells. After transfection, infectivity, expressed as pfu, was determined by plating on permissive *B. subtilis* Su^+44 cells.

measured as the ability to produce phage ϕ29 particles, was compared with that of the natural (*in vivo*) ϕ29 DNA. Thus, similar amounts of ϕ29 DNA, either obtained from ϕ29 *sus14* (1242) virions or by *in vitro* amplification of ϕ29 *sus14* (1242) DNA, were used to transfect *B. subtilis* competent cells, and the number of infective centers was determined by plating on permissive (suppressor) *B. subtilis* cells. As shown in Fig. 5, infectivity of both natural (*in vivo*) and synthetic (*in vitro*) ϕ29 DNA was virtually identical; the transfection efficiency was $\approx 10^4$ pfu/μg. Moreover, the size distribution of individual plaques was also identical, indicating the absence of *in vitro* mutations affecting phage growth. These results indicate that the *in vitro* ϕ29 DNA amplification system produces mature molecules and also suggest that DNA synthesis is being carried out with high fidelity. This could be expected if one takes into account previous estimations of the insertion fidelity of ϕ29 DNA polymerase (28) and its strong $3' \rightarrow 5'$ exonuclease activity (29, 30). A minimal estimate of the fidelity of this amplification system is the fact that no plaques were obtained when the transfection mixture corresponding to the *in vitro*-amplified ϕ29 DNA was plated in nonpermissive *B. subtilis* cells. This was the expected result if the nonsense mutation present in the input ϕ29 DNA was maintained in the *in vitro*-amplified ϕ29 DNA molecules.

DISCUSSION

In this paper, one of the most efficient *in vitro* DNA replication systems described so far is presented. By using four ϕ29 DNA replication proteins—TP (primer), DNA polymerase, protein p6 (DBP), and protein p5 (SSB)—it has been possible to amplify the 19,285-bp-long ϕ29 DNA molecule by three orders of magnitude after 1 hr of incubation at 30°C. As it occurs during ϕ29 DNA replication *in vivo*, each of these proteins was an absolute requirement. The biological activity of the *in vitro*-amplified ϕ29 DNA was demonstrated by its ability to produce phage ϕ29 particles when transfected into *B. subtilis* cells.

It was expected that, under the different conditions used, the amount of DNA synthesized (amplified) would largely depend on the efficiency of initiation and the frequency of reinitiations both on the same and/or different DNA molecules. Moreover, elongation was not expected to be rate-

limiting because once a molecule is initiated, complete elongation could be achieved in about 5 min (9). However, size analysis of the synthesized DNA indicates that at a high ratio among initiation factors (TP, DNA polymerase, and DBP) and input DNA (replication origins), there is a blockage of elongation that can be only prevented by addition of the SSB protein. This blockage could be due to a "mechanical" hindrance produced by the multiple displaced strands originated from frequent reinitiations at the same origin.

A variety of nucleic acid amplification techniques, developed as tools for nucleic acid analysis and manipulation, are also being successfully applied for clinical diagnosis of genetic and infectious diseases. Amplification techniques (reviewed in ref. 31) can be grouped into (*i*) those requiring temperature cycling (PCR and ligase chain reaction) and (*ii*) isothermal systems [transcription-based amplification systems (3SR and NASBA), strand-displacement amplification, and Qβ replication systems]. Two aspects are frequent caveats in these procedures: fidelity of synthesis and length of the amplified product. As it has been recently reported, the use of proofreading polymerases (containing 3′ → 5′ exonuclease activity) in combination with 3′ → 5′ exo-deficient enzymes significantly increases not only fidelity but also the size limit of the amplification reaction (32, 33).

The results presented in this paper establish basic requirements for the development of an amplification system relying on the mechanism of phage ϕ29 DNA replication. This system would be adequate for faithful amplification of DNA molecules longer than 70 kb (9), largely over the size limit obtained with the amplification systems available to date. This procedure of isothermal TP-primed amplification would exploit the peculiar properties of ϕ29 DNA polymerase: (*i*) ability to use a protein as primer, (*ii*) intrinsic high processivity (>70 kb), and (*iii*) strand displacement coupled to DNA synthesis. This procedure would also require the action of the viral DBP, to efficiently recognize and activate the ϕ29 DNA replication origins (which should be present at the ends of the target DNA to be amplified), and SSB, to allow maturation of replicative intermediates and reinitiation on new DNA molecules. The sequences at ϕ29 DNA ends that are active as minimal replication origins (34) and the location of the protein p6 nucleation site absolutely required for protein p6 binding and activation of the ϕ29 DNA replication origins (3) have been determined. Therefore, DNA molecules suitable for TP-primed amplification could be directly obtained by ligation of double-stranded DNA fragments containing the ϕ29 DNA replication origins to both ends of the DNA molecule to be amplified. Alternatively, selective (targeted) amplification would require initial stages of single-stranded DNA priming based on the use of dual primers (specific sequence plus ϕ29 DNA origin sequence) to obtain specific double-stranded DNA linear molecules flanked by ϕ29 DNA replication origins. Once these molecules are formed and the ϕ29 DNA replication proteins are provided, initiation, progression, and resolution steps would occur continuously, producing an amplification cascade similar to that occurring during viral infection.

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