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Nucleotide sequence at the termini of the DNA of *Bacillus subtilis* phage ϕ29

(DNA sequencing/initiation of replication/protein–DNA interaction)

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ABSTRACT **Phage ϕ29 DNA cannot be phosphorylated with polynucleotide kinase and [γ-^{32}P]ATP because of the presence of a viral protein covalently linked to the 5′ termini. The 5′ ends can, however, be made susceptible to phosphorylation by treatment with alkali and alkaline phosphatase. Restriction fragments *Hpa* II C and *Hpa* II F, corresponding to the right and left ends of ϕ29 DNA, respectively, were labeled at the 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP or at the 3′ ends with terminal transferase and [α-^{32}P]ATP or [α-^{32}P]cordycepin 5′-triphosphate. After a secondary cleavage of the labeled fragments, the sequence of the first 150–180 nucleotides at the termini of ϕ29 DNA was determined by the method of Maxam and Gilbert. The ends of ϕ29 DNA are flush, and a six-nucleotides-long inverted terminal repetition was found. The functional implications of the sequences determined are discussed.**

The genome of *Bacillus subtilis* phage ϕ29 is a double-stranded, linear DNA molecule (M_r 11,800,000) (1) that has a protein covalently linked to the two 5′ termini (2–5). The protein bound to the DNA is the product of cistron 3, p3 (2), an early protein (M_r 27,000) (6, 7) required for the initiation of ϕ29 DNA replication (8). The DNA of adenoviruses, which also have a protein linked to the 5′ termini (9, 10), contains an inverted terminal repetition of ≈100 nucleotides (11–16). Two observations suggest that such an extensive inverted terminal repetition does not exist in ϕ29 DNA: (*i*) single-stranded circles were not found after denaturation of the DNA followed by renaturation at low concentrations (A. Talavera, personal communication) and (*ii*) the degree of melting of the two ends of ϕ29 DNA was different in a partial denaturation map (17).

Initiation of both adenovirus (18–22) and ϕ29 (23, 24) DNA replication occurs at or close to either DNA end, nonsimultaneously, and proceeds by a mechanism of strand displacement. Models for the initiation of adenovirus and ϕ29 DNA replication have been proposed in which a newly synthesized molecule of the terminal protein acts as a primer, providing the 3′-OH group needed for elongation by the DNA polymerase (9, 18, 23, 24). An alternative model for initiation of replication by a linear DNA that cannot form circles or concatemers involves the formation of a hairpin loop at the 3′ ends of the DNA, which thus provides the 3′-OH group needed for elongation (25). This model has been ruled out in the case of several types of adenovirus DNAs, because no palindromic sequences consistent with the foldback of the 3′ ends have been detected within the 150–200 nucleotides sequenced at the two DNA ends (11, 12) or even within the terminal 3% at the right-hand end (26).

We report the sequence of the first 150–180 nucleotides at the ends of ϕ29 DNA and show that (*i*) ϕ29 DNA termini are flush; (*ii*) there is a short inverted terminal repetition, six nucleotides long; and (*iii*) no sequence consistent with a hairpin model for initiation of replication exists within the first nucleotides sequenced at either DNA end. A preliminary account of some of these findings has been presented (27).

MATERIALS AND METHODS

Nucleoside Triphosphates and Enzymes. [γ-^{32}P]ATP was prepared essentially by the method of Schendel and Wells (28). Five millicuries (1 Ci = 3.7×10^{10} becquerels) of carrier-free $^{32}P_i$ were added for a 0.05-ml reaction volume. [α-^{32}P]ATP (400 Ci/mmol) and [α-^{32}P]dCTP (410 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England). [α-^{32}P]cordycepin-5′-triphosphate (537 Ci/mmol) was from New England Nuclear. Polynucleotide kinase, *Hpa* II, *Hin*dIII, *Taq* I, and *Hha* I were obtained from New England Biolabs; terminal transferase was from Bethesda Research Laboratories (Rockville, MD) or P-L Biochemicals; bacterial alkaline phosphatase was from Worthington; alkaline phosphatase, from calf intestine and DNA polymerase (Klenow fragment) were from Boehringer Mannheim; and fungal proteinase K, chromatographically purified, was from Merck.

Preparation of ϕ29 DNA and Restriction Fragments. DNA was isolated from the phage ϕ29 delayed-lysis mutant *sus*14(1242) by treatment with proteinase K as described (29). After phenol extraction, the DNA was precipitated with 2.5 vol of ethanol.

DNA restriction fragments were separated by electrophoresis on 3.5% or 5% polyacrylamide gels, 20 × 20 or 40 × 0.2 cm, in 36 mM Tris·phosphate, pH 7.8/1 mM EDTA (30). The electrophoresis was run at room temperature at a constant voltage of 5–7.5 V/cm. The maximum amount of DNA loaded on a gel was ≈150 μg/0.4 cm^2. DNA fragments were also separated on 1% agarose analytical horizontal gels, 30 × 12 × 0.4 cm, in 40 mM Tris·acetate, pH 7.8/1 mM EDTA at a constant voltage of 3.33 V/cm.

The DNA fragments were located by autoradiography and recovered from the crushed polyacrylamide gel slices by extraction with an excess volume of 50 mM Tris·HCl, pH 7.8/2 mM EDTA/0.15 M NaCl. The DNA was purified by adsorption on a DEAE-cellulose column (≈0.3-ml bed volume), followed by elution with 1.5 ml of 50 mM Tris·HCl, pH 7.8/2 mM EDTA/1.5 M NaCl. The DNA was recovered by precipitation with 2.5 vol of ethanol.

Alkali Treatment of ϕ29 DNA and Restriction Fragments. Deproteinized ϕ29 DNA or restriction fragments were treated with 0.1 M NaOH at 37°C for 1.5 hr at a DNA concentration of 15–80 μg/ml. To reanneal the DNA, the solution was neutralized by addition of an equal volume of 0.11 M HCl/1 mM Tris·HCl, pH 7.8/2 mM EDTA, heated in a bath of boiling water for 7 min, and incubated at 69°C for 4 hr in the presence of 0.25 M NaCl.

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Abbreviation: bp(s), base pair(s).

Labeling the Ends of ϕ29 DNA Fragments and DNA Sequencing. ϕ29 DNA restriction fragments were labeled at their 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP essentially by the procedure of Maxam and Gilbert (31). The DNA was dephosphorylated by digestion for 1 hr at 37°C with either bacterial alkaline phosphatase in 50 mM Tris·HCl, pH 8 (85 μg of enzyme/pmol of phosphorylated end) or alkaline phosphatase from calf intestine in 20 mM Tris·HCl, pH 9.5/0.1 mM EDTA/1 mM spermidine (35 μg of enzyme/pmol of phosphorylated end). The bacterial enzyme was removed by phenol extraction. The calf intestine enzyme was inactivated by heating at 69°C for 2 hr (32). The labeling reaction with polynucleotide kinase was stopped by freezing and thawing twice, and the DNA was separated from the unreacted ATP by column chromatography on Sephadex G-50. By this procedure, terminal restriction fragments of ϕ29 DNA are labeled only at one 5′ end, because the 5′ termini of ϕ29 DNA are blocked by protein p3. To label the 5′ ends of ϕ29 DNA, protein p3 was removed by treatment with alkali as described, and the product was dephosphorylated with phosphatase.

ϕ29 DNA restriction fragments were labeled at their 3′ ends with terminal deoxynucleotidyl transferase and [α-^{32}P]ATP essentially by the Maxam and Gilbert procedure (31, 33). After treatment with NaOH to remove all the AMP residues added except the first one, the DNA was reannealed as described above. Alternatively, ϕ29 DNA restriction fragments were labeled at their 3′ ends with terminal deoxynucleotidyl transferase and [α-^{32}P]cordycepin-5′-triphosphate according to Tu and Cohen (34). Labeling of the terminal *Taq* I fragments at only the 3′ end of the *Taq* I site was carried out by incubation with DNA polymerase (Klenow fragment) and [α-^{32}P]dCTP at 6°C (35).

DNA sequencing was done essentially by the Maxam and Gilbert method (31). The slab gels were 400 × 200 or 300 × 0.5–1 mm and had 10-mm slots. Twenty per cent polyacrylamide gels were used for sequencing the first 20 nucleotides, and 8% polyacrylamide gels were used for sequencing the nucleotides that were further from the labeled end. In some experiments, the hydrazine was eliminated by centrifugation through Sephadex G-50 (36). Autoradiography was carried out with Ilford fast tungstate intensifying screens at −70°C, when needed.

RESULTS

Labeling the 5′ Ends of ϕ29 DNA. Proteinase K-treated ϕ29 DNA cannot be phosphorylated by polynucleotide kinase (2), probably because a small peptide remains linked to the DNA (37). The linkage of the protein to the DNA is sensitive to alkali (2). We found that treatment with 0.1 M NaOH for 1.5 hr at 37°C, followed by digestion with alkaline phosphatase, unblocks the 5′ termini of ϕ29 DNA and makes them susceptible to phosphorylation by polynucleotide kinase. The pattern of fragments obtained when an *Hpa* II digest of ϕ29 DNA was treated with alkaline phosphatase and then labeled with polynucleotide kinase and [γ-^{32}P]ATP is shown in Fig. 1*A*. The two *Hpa* II fragments from the termini of ϕ29 DNA, fragments C and F (unpublished results; ref. 38), were labeled with about half the efficiency of the other internal fragments. When the peptides blocking the 5′ termini were released by treatment with alkali but the DNA fragments were not treated with alkaline phosphatase, there was only a residual labeling of all the fragments (Fig. 1*B*), probably because of an exchange reaction catalyzed by the polynucleotide kinase (39). However, when the DNA was dephosphorylated with alkaline phosphatase after treatment with alkali and then labeled with polynucleotide kinase and [γ-^{32}P]ATP, all the fragments, even those located at the ends of ϕ29 DNA, were labeled with approximately the same efficiency (Fig. 1*C*).

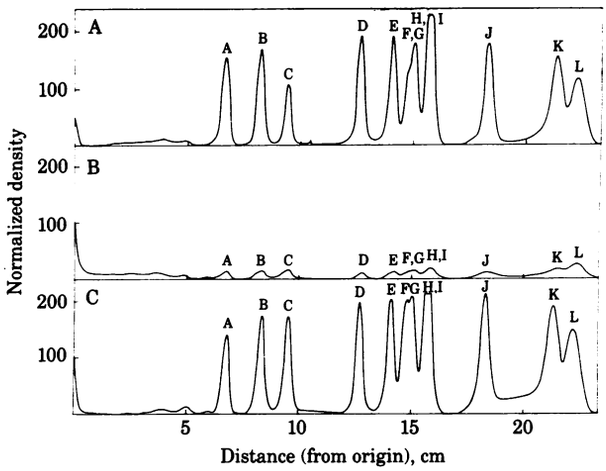

FIG. 1. Densitometry of electrophoretic separation of *Hpa* II fragments of ϕ29 DNA labeled with polynucleotide kinase and [γ-^{32}P]ATP after treatment with alkali or phosphatase or both. Fifty μg of ϕ29 DNA were digested with *Hpa* II in a final volume of 75 μl. (*A*) Twenty-five μl of the *Hpa* II digest were precipitated with 2.5 vol of ethanol, treated with alkaline phosphatase, and labeled with polynucleotide kinase and [γ-^{32}P]ATP. The labeled material was precipitated with ethanol, dissolved in electrophoresis buffer diluted 1:20, and loaded on a 1% agarose gel slot (0.5 × 0.4 cm). (*B*) Fifty μl of the *Hpa* II digest were treated with alkali. After reannealing, the DNA was precipitated with ethanol, and the precipitate dissolved in phosphatase buffer and half sample was labeled with polynucleotide kinase and [γ-^{32}P]ATP without prior treatment with alkaline phosphatase. The labeled material was processed as above. (*C*) Half of the *Hpa* II digest that had been treated with alkali as above was treated with alkaline phosphatase and labeled with polynucleotide kinase and [γ-^{32}P]ATP. The labeled material was processed as above. Letters above the peaks indicate the names of the DNA restriction fragments as given by Ito and Kawamura (38). The last peak is a new, small fragment recently found (unpublished results).

When the two labeled terminal *Hpa* II fragments, C and F, were digested with a second restriction enzyme—i.e., *Hin*dIII for the right-hand end fragment, C, and *Hha* I for the left-hand end fragment, F—two labeled fragments were obtained when the DNA had been labeled after treatment with alkali and phosphatase and only one was obtained when the DNA was labeled after dephosphorylation with phosphatase without previous treatment with alkali (Fig. 2). Fragments L (from the *Hin*dIII digestion) and O (from the *Hha* I digestion), not labeled unless the terminal peptide had been eliminated by alkali treatment of fragments C and F, respectively (see Fig. 2), have been mapped at the ends of ϕ29 DNA as described below.

Alkali treatment was also used to label the 5′ ends of adenovirus DNA (10, 14, 16).

Isolation of Terminal DNA Fragments Labeled Only at the Ends of the ϕ29 Genome. After treatment with alkali, intact ϕ29 DNA cannot be reannealed completely in a correct way and more than 50% of it behaves as a high molecular weight complex that does not enter a 1% agarose gel (data not shown). Because alkali treatment had been used to remove protein p3 from the 5′ termini of ϕ29 DNA and also when the 3′ ends were labeled with [α-^{32}P]ATP and terminal transferase, we used as starting material fragments C and F from the *Hpa* II digestion, which proved to be easier to reanneal. Right-hand and left-hand end fragments, C and F, respectively, labeled at their 5′ or 3′ ends, were cut with a second restriction enzyme, *Hin*dIII for fragment C, giving rise to terminal fragment L (38), about 250 nucleotides long, and *Hha* I for fragment F, which produced fragment O,

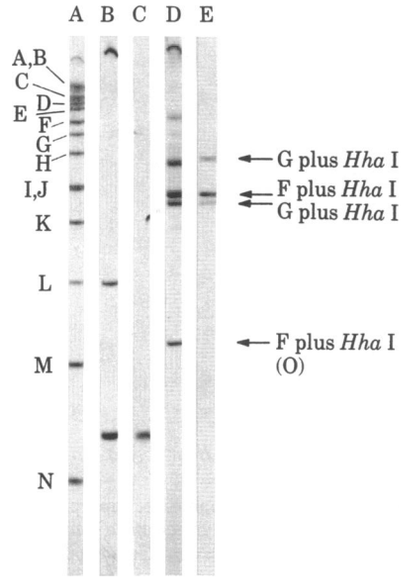

FIG. 2. Autoradiograph of an electrophoretic separation on 5% polyacrylamide gel. (*A*) ϕ29 DNA was cleaved with *Hin*dIII and the products were labeled with polynucleotide kinase and [γ-^{32}P]ATP after treatment with alkaline phosphatase. Letters on the left indicate the names of the DNA restriction fragments as given by Ito and Kawamura (38). (*B*) Fragment *Hpa* II C was labeled with polynucleotide kinase and [γ-^{32}P]ATP after treatment with alkali and alkaline phosphatase and then digested with *Hin*dIII. (*C*) Fragment *Hpa* II C was treated as above, except that the alkali treatment was omitted. (*D*) Fragments *Hpa* II F and G were labeled with polynucleotide kinase and [γ^{32}P]ATP after treatment with alkali and alkaline phosphatase and then digested with *Hha* I. Fragments F and G have very similar mobilities in the preparative 3.5% polyacrylamide gel and they were taken together. The high molecular weight band seen here is probably due to undigested fragments F and G. (*E*) Fragments F and G were treated as above, except that the alkali treatment was omitted.

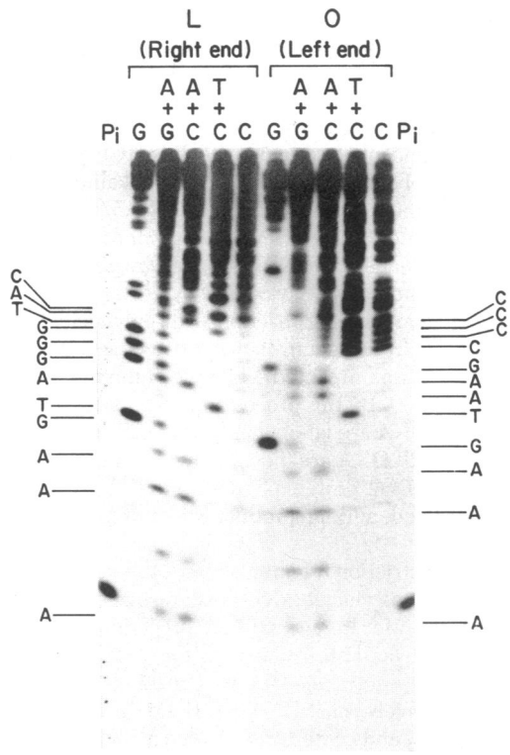

FIG. 3. Determination of the sequence of the first 12 nucleotides at both 5′ ends of ϕ29 DNA. Fragments *Hin*dIII L and *Hha* I O, uniquely labeled at the 5′ ends corresponding to the termini of the ϕ29 genome, were subjected to the chemical reactions of the Maxam and Gilbert sequencing method (31) and electrophoresed through a 20% polyacrylamide gel containing 8.3 M urea. $^{32}P_i$ was loaded in parallel slots as marker.

about 170 nucleotides long (see Fig. 2). Fragment O was shown to be the terminal one, because its electrophoretic mobility was identical to that of one of the two terminal fragments obtained by treatment of ϕ29 protein–DNA complex with *Hha* I, which were characterized by their retention on Millipore filters due to the presence of the 5′-linked protein (data not shown).

Determination of the Nucleotide Sequence at the Ends of ϕ29 DNA. The sequence of the first 150–180 nucleotides at the termini of ϕ29 DNA was determined by using as substrates for the chemical degradation reactions, the terminal fragments L and O, uniquely labeled at the 5′ or 3′ ends of the ϕ29 genome. The sequencing gel for the first nucleotides at the 5′ ends of ϕ29 DNA is shown in Fig. 3. $^{32}P_i$ was run in parallel slots to determine the first nucleotide at the ends of the DNA—i.e., the one that carries the ^{32}P label. ϕ29 DNA that had been digested with *Hin*dIII was labeled at the 5′ ends, sequenced, and run in parallel in some experiments as a control for the position of the first bands (data not shown). This comparison suggested that the band that appears in Fig. 3 between the first and second A is an artifact, probably a product of incomplete β elimination. Therefore, there is a six-nucleotide-long sequence, AAAGTA, identical at the two 5′ ends of ϕ29 DNA (see Fig. 3). This sequence was confirmed by analysis of the two terminal fragments produced by *Taq* I cleavage of ϕ29 DNA, 53 and 44 nucleotides long, labeled at the 3′ ends of the *Taq* I site with DNA polymerase as described above. That the first nucleotide at the termini is adenine agrees with the finding by Hermoso and Salas (37) that the nucleotide linked to protein p3 is dAMP. The sequences of the first 150 and 180 nucleotides at the left and right termini, respectively, of ϕ29 DNA are shown in Fig. 4. All the sequences were determined from both the 5′ and 3′ ends of the two DNA termini. In addition, the sequence of the first nucleotides at the two 3′ termini was obtained from the terminal *Taq* I-digestion fragments labeled at the 5′ ends of the *Taq* I site.

DISCUSSION

Initiation of replication of both adenovirus and phage ϕ29 DNAs occurs at or close to either DNA end (18–24). It seems likely that the nucleotide sequence at the termini of these DNAs would contain some signal for the initiation of replication. The sequences at the ends of several adenovirus DNAs have been determined. In all cases, an inverted terminal repetition of $\approx$100 nucleotides has been found (11–16), and this sequence has been well conserved in adenovirus evolution, especially the first 50 base pairs (14). In ϕ29 DNA, we find a shorter inverted terminal repetition of six nucleotides, which is probably long enough to be recognized by the protein(s) involved in initiation of DNA replication. In fact, the major region of homology between the different types of adenovirus DNAs is 14 nucleotides long (14, 16), and the two critical regions in promoters recognized by *Escherichia coli* and *B. subtilis* RNA polymerases in procaryotes are 6–12 nucleotides long (40, 41).

An important problem in the replication of a linear DNA, such as that of the adenoviruses and phage ϕ29, is how initiation of replication takes place at the ends. How is the free 3′-OH group required by all known DNA polymerases provided and how does this allow full copying of the DNA. The formation of

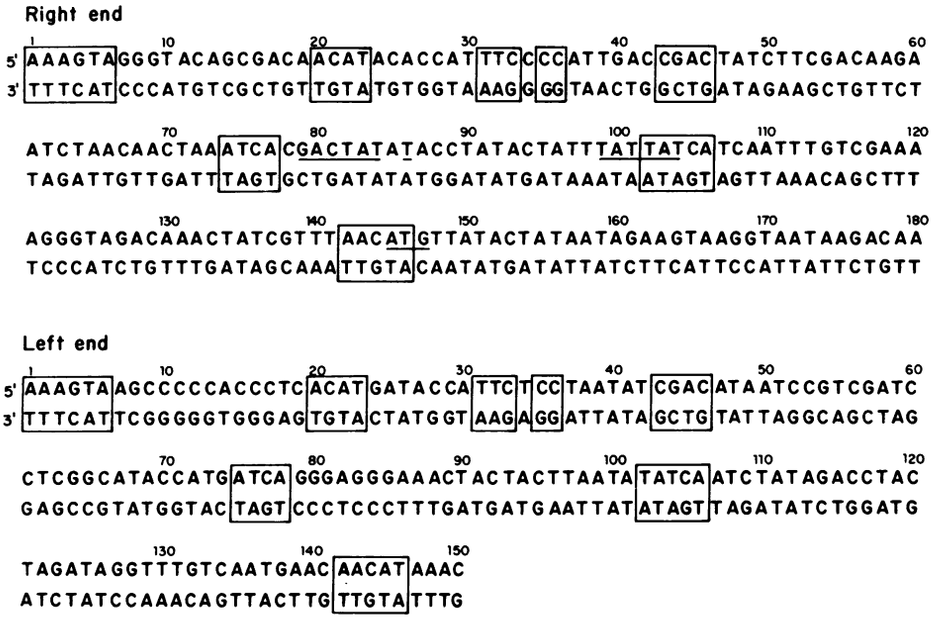

FIG. 4. Sequences of the first 150–180 nucleotides at the ends of ϕ29 DNA. Sequences in boxes show the homologies between the two ends. The sequences underlined are promoter-like sequences (−35 region and Pribnow box) and a potential initiation codon for protein synthesis.

intracellular covalent circles or concatemers, a way by which this problem is sometimes solved, has not been detected in ϕ29 DNA replication (23, 24). Also, as in the case of adenovirus DNAs (11, 12, 26), the possibility of formation of a hairpin loop at the 3′ ends of ϕ29 DNA is ruled out.

A model has been proposed, for both adenovirus (9, 18) and ϕ29 (23, 24) DNA replication, in which a newly synthesized molecule of the terminal protein, by recognizing the parental terminal protein, the inverted terminal repetition, or both, would be positioned at the ends of the DNA and, by reaction with the initiating deoxynucleoside triphosphate and formation of a protein-deoxynucleoside monophosphate covalent linkage would provide the 3′-OH group needed. The fact that terminal protein has been found at all the ends of replicating molecules both in adenoviruses (42–45) and ϕ29 DNA, (unpublished results) supports this model. The linkage between the terminal protein and the DNA has been shown to be a phosphoester bond between the OH group of a serine residue and 5′-dAMP or 5′-dCMP for phage ϕ29 (37) and adenovirus 5 (46), respectively. If this model is correct, it still remains to be determined when and how the linkage between the terminal protein and the deoxynucleoside monophosphate is produced—i.e., whether the terminal protein binds to the initiating nucleotide before or after being positioned at the ends of the DNA and whether the terminal protein itself or some other virus coded or cellular protein catalyzes the reaction.

In one of the models for adenovirus DNA replication, the inverted terminal repetition is also involved in the cyclization of the displaced parental single strand, to initiate its replication, although this is not considered an essential feature (18). In the case of ϕ29 DNA, this type of circularization is not likely to occur because of the short and adenine- and thymine-rich inverted terminal repetition. As is shown in Fig. 4, there are also other small inverted repetitions all along the first 150 nucleotides. In fact, there is a quite high degree of homology between the sequences of the two ends of ϕ29 DNA, 48% homology for nucleotides 1–50, and 40% for nucleotides 1–150. It remains to be determined whether those similarities have any significance.

Terminal sequences possibly recognized by enzymes involved in nucleic acid replication have been also reported in reovirus 3. In this case, several segments of the reovirus genome contain 3′ common sequences that are thought to be recognized by the viral transcriptase and replicase activities for the synthesis of mRNAs and minus strands (47).

No homology exists among the sequences at the ends of ϕ29 DNA and those of adenovirus DNAs (11–16). This is not surprising; presumably the proteins involved in the initiation of replication of ϕ29 and adenovirus DNAs are different despite the fact that the two systems probably initiate replication by similar mechanisms. Nevertheless, there is one important common feature of the inverted terminal repetition of ϕ29 DNA and the end proximal 14 base pairs [bp(s)] common to different adenovirus DNAs (11–16), the fact that they are very rich in adenine-thymine pairs. Such regions are also present in the origins of replication of *E. coli*, λ, and G4 DNAs (48–50), although, in these cases, the whole mechanism of initiation of replication is different from that in ϕ29 DNA. It seems likely that the presence of regions rich in adenine-thymine pairs is important in DNA sites where a local melting of DNA is needed, as is the case at the origin of replication.

It has been suggested that adenoviruses (51, 52) and *B. subtilis* phages (53) contain short single-stranded regions at the 5′ termini of the DNA. In ϕ29 DNA, the determination of the sequence of the two strands at both ends of the DNA indicates that they are flush.

A strong binding site for RNA polymerase at 126 ± 72 bp from the right-hand end has been mapped by electron microscopy (1). The average sequences recognized by the *E. coli* RNA polymerase at the −35 region and the Pribnow box are T-G-T-T-G-A-C-A-A-T-T-T-*13 bp*-T-A-T-R-A-T-G-*5 bp* start transcription (41) and, for an early promoter of phage SP01, recognized by *B. subtilis* RNA polymerase, the sequences found were T-T-G-A-C-T-*17 bp*-C-A-T-A-A-T-*6 bp* start transcription (40). Possible sequences for the −35 region and the Pribnow box at the right end of ϕ29 DNA are underlined in Fig. 4. In agreement with other promoters recognized by bacterial RNA polymerases (40), the regions between nucleotides −30 to −20

and −12 to +3 are very rich in adenine and thymine (72% the first region, 86% the latter). At position 145, 35 nucleotides after the first nucleotide of the hypothetical RNA (assuming that the initiation of RNA synthesis takes place 5–6 nucleotides after the underlined Pribnow box), a potential initiation codon AUG is found. This is the only AUG found within the 180 nucleotides sequenced and is probably the initiation codon for protein 17 (54). No termination codon in phase with this AUG is found in the next 32 nucleotides sequenced. Sequences complementary to the 3′ end of the 16S rRNA of *B. subtilis* [3′-U-C-U-U-U-Pyr(X)$_7$G (55)] can be found in the nucleotides preceding this AUG, around position 120 or 130.

The nucleotide sequences at the termini of ϕ29 DNA have been independently obtained by Yoshikawa *et al.* (56); the results are similar.

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