DNA replication in the halophilic archaeobacterium *Haloferax volcanii* in the absence of protein synthesis

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In *Haloferax volcanii*, DNA replication is not arrested in the absence of protein synthesis. This DNA replication occurs either in complete medium during the inhibition of protein synthesis by anisomycin or in minimal medium during amino acid starvation of an auxotrophic mutant *his-1*. Once established, this DNA synthesis is permanent. It is also sensitive to aphidicolin, an inhibitor of eukaryotic chromosomal DNA replication. The entire chromosome seems to participate in this replication whereas the synthesis of the resident pHV2 plasmid is reversibly inhibited.

Introduction

Chromosomal DNA replication is a key event in the life of all organisms and, consequently, considerable effort has been expended to understand it. In the best studied member of the eubacteria, *Escherichia coli*, DNA replication has been shown to be controlled mainly at the level of initiation (for reviews, see McMacken *et al.*, 1987 and von Meyenburg & Hansen, 1987). *In vivo* studies have revealed that protein synthesis is required for the reinitiation of new rounds of DNA replication but not for completing on-going ones. Inhibition of protein synthesis results in a complete cessation of DNA synthesis after the termination of on-going rounds of DNA replication (Lark *et al.*, 1963; Maaloe & Hanawalt, 1961).

In archaeobacteria, members of a third cell lineage (Torreblanca *et al.*, 1986; Woese & Olsen, 1986), our knowledge of the control of DNA replication is extremely limited and restricted to the characterization of certain DNA polymerases (Ben Mahrez *et al.*, 1991; Elie *et al.*, 1989; Hamal *et al.*, 1990; Klimczak *et al.*, 1985a, b; Kohiyama *et al.*, 1986; Nakayama & Kohiyama, 1985; Nakayama *et al.*, 1988; Salhi *et al.*, 1990; Zabel *et al.*, 1985). Here, we examine the effect on DNA synthesis of

amino acid starvation and inhibition of protein synthesis by anisomycin in the aerobic chemo-organotroph *Haloferax volcanii* (Torreblanca *et al.*, 1986).

Members of the genus *Haloferax* are only moderately halophilic, requiring 2–3 M-NaCl for optimal growth, and are capable of growth on single carbon sources. We have chosen to study an auxotrophic mutant of *Haloferax volcanii*, in which we previously reported the continuation of DNA synthesis during amino acid starvation (Sorokine & Kohiyama, 1988).

In this report, we show that replication of the chromosomal DNA but not of the resident pHV2 plasmid (Charlebois *et al.*, 1987) is independent of protein synthesis. Continuous DNA synthesis occurs either when a required amino acid is removed or when the protein synthesis inhibitor anisomycin is added. The observed DNA synthesis does not need to be induced, and all the chromosomal DNA but not the pHV2 plasmid of *H. volcanii* is apparently replicated. The inhibition of pHV2 plasmid replication is a reversible event.

Methods

Chemicals and radioisotopes. Aphidicolin, anisomycin, histidine and 4',6-diamidino-2-phenylindole·2HCl (DAPI) were obtained from Sigma. Kanamycin was from Appligène laboratories. [α -³²P]dTTP and [α -³²P]dATP [3000 Ci (110 TBq) mmol⁻¹], [methyl-³H]thymidine [44 Ci (1628 GBq) mmol⁻¹], [methyl-¹⁴C]thymidine [25 mCi (925 mBq) mmol⁻¹] and [³H]L-alanine [59 Ci (2183 GBq) mmol⁻¹] were obtained from Amersham. *Xho*I and *Pst*I were from Boehringer Mannheim.

Bacteria. The bacterial strain used is an auxotrophic *his-1* mutant of *Haloferax volcanii* DS2. This mutant, WR201, was provided by Dr Mevarech, University of Tel Aviv, Israel (Mevarech & Werczberger, 1985).

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Abbreviation: DAPI, 4',6-diamidino-2-phenylindole·2HCl.

Fig. 1. Effect of histidine deficiency on bacterial growth, DNA replication and protein synthesis in a *his-1* mutant of *H. volcanii*. An exponential culture of the *his-1* mutant in minimal medium supplemented with histidine (MM⁺) was washed in minimal medium without histidine (MM⁻) at OD₆₀₀ = 0.1 and divided into two portions. Histidine was added to one at 0 h and this culture served as control. L-[³H]Alanine (1.4 μCi ml⁻¹) was then added to both cultures in the presence of 1 mM-L-alanine and incubation was continued for the indicated times at 37 °C. Results are shown for cultures grown in MM⁺ (▲, ▼) and in MM⁻ (●, ■). (a) Culture during exponential growth: OD₆₀₀ (log scale) in MM⁺ (▲) and radioactive labelling (linear scale) of bacteria (▼). Culture during amino acid starvation: OD₆₀₀ in MM⁻ (●) and radioactive labelling of bacteria with L-[³H]alanine (■). (b) Culture during exponential growth: OD₆₀₀ in MM⁺ (▲) and DNA content measured by fluorometry using the DAPI method (▼). Culture during amino acid starvation: OD₆₀₀ in MM⁻ (●) and DNA content measured by fluorometry using the DAPI method (■). The arrow indicates the time of readdition of histidine to MM⁻ cultures.

Media, growth conditions and amino acid starvation. Bacteria were grown in a salt mixture containing (l⁻¹) 206 g NaCl, 36.9 g MgSO₄·7H₂O, 5 ml 1 M-KCl, and 1.7 ml of a 75 mg l⁻¹ solution of MnCl₂. For complete medium (CM), 5 g yeast extract (Difco) l⁻¹ was added. When minimal medium (MM) was prepared, the following components were added: 5 ml 1 M-NH₄Cl, 45 ml 10% (v/v) glycerol, 5 ml 10% (w/v) succinate, 1 ml trace element solution (30 mg MnCl₂, 44 mg ZnSO₄, 230 mg FeSO₄, and 5 mg CuSO₄ per 100 ml). In each case, after the medium had been autoclaved and cooled, 5 ml 10% (w/v) CaCl₂·2H₂O and 2 ml 0.5 M-K₂HPO₄ were added; the pH was then adjusted to 7.2 (Mevarech & Werczberger, 1985). When needed, histidine (final concn, 50 μg ml⁻¹) was added to minimal medium (MM⁺); the medium without histidine was denoted MM⁻. For agar plates, 25 g Bacto-Agar (Difco) l⁻¹ was added. Bacteria were grown at 37 °C with shaking. Kanamycin was included in CM, MM⁺ and MM⁻ at 50 μg ml⁻¹ to avoid contamination with halotolerant eubacteria. Growth was followed by measuring OD₆₀₀.

For kinetic experiments, freshly inoculated cultures were shaken at 37 °C until the cells had reached an OD₆₀₀ of 0.1–0.15 (corresponding to a viable count of 1.2–2.5 × 10⁸ c.f.u. ml⁻¹). The cells were then centrifuged (6000 g, 12 °C, 15 min), washed with two vols CM (to remove antibiotics) or MM⁻ (to remove histidine), and resuspended in the same volume of CM or MM (supplemented or not with histidine). At this point chemicals and radioisotopes were added.

Determination of DNA replication by radioactive labelling of DNA or fluorometric determination using DAPI. For the determination of DNA concentration, the *his-1* mutant culture was radioactively labelled with [methyl-³H]thymidine (0.5 μCi ml⁻¹) in the presence of thymidine (4 μg ml⁻¹) in MM, or [methyl-³H]thymidine alone (1 μCi ml⁻¹) in CM. Samples were removed at different times, the DNA was precipitated by addition of ice-cold 5% (w/v) trichloroacetic acid (TCA) containing 0.02 M-sodium pyrophosphate and collected on Whatman GFB filters. The filters were dried and the radioactivity was determined in a scintillation counter. Fluorometric determination of total DNA per cell

was performed according to the technique described by Legros & Kepes (1985) and modified as follows: samples of bacteria (300 μl) were mixed with toluene (3%, v/v), shaken vigorously and stored at -40 °C. Then, toluenized bacterial samples were thawed, and 2.7 ml of a DAPI solution (90 μg l⁻¹) was added to each sample. Measurements of DNA-DAPI complexes were made on a Jobin Yvon JY3D fluorometer. A blank was prepared with culture medium. Calf thymus DNA diluted in MM served as a standard.

Determination of protein synthesis. The bacteria were radioactively labelled with L-[³H]alanine (1.4 μCi ml⁻¹) in the presence of L-alanine (1 mM). Samples were removed at different times and proteins were precipitated with ice-cold 5% TCA. Samples were then filtered through Whatman GFB filters, rinsed with ice-cold TCA, dried, and the radioactivity determined in a scintillation counter.

Pulse-labelling of DNA. The bacterial cultures in MM⁺ or MM⁻ were pulse-labelled with 0.5 μCi [methyl-¹⁴C]thymidine ml⁻¹ for approximately half the generation time. We have observed that [α-³²P]dTTP also labels *H. volcanii* DNA so, in another experiment, we labelled cells for 1 h with 2 μCi [α-³²P]dTTP during exponential growth or amino acid starvation. Then, cells were collected by centrifugation at 6000 g for 15 min, washed with MM⁻ and lysed by resuspension in TE buffer [10 mM-Tris/HCl, 1 mM-EDTA (pH 7.5)]. The samples were then treated with RNAase A (30 μg ml⁻¹) for 30 min at 37 °C, followed by proteinase K (100 μg ml⁻¹) treatment for 2 h at 37 °C before phenol/chloroform extraction and ethanol precipitation (Maniatis *et al.*, 1982).

DNA was digested with *Pst*I or *Xho*I and subjected to 0.8% agarose gel electrophoresis. The gels were dried and autoradiographed.

Preparation of the pHV2 plasmid DNA probe. The plasmid pHV2 of *H. volcanii* (Charlebois *et al.*, 1987) was isolated by CsCl/ethidium bromide buoyant density ultracentrifugation (Maniatis *et al.*, 1982), digested with *Pst*I and subjected to 0.8% agarose gel electrophoresis. After electroelution from the gel, pHV2 DNA was labelled with [α-

^{32}P dATP using the multiprime DNA labelling system as described by the supplier (Amersham).

Southern blot analysis. DNA was digested with various restriction enzymes according to the conditions suggested by the manufacturer (Boehringer). Agarose gel electrophoresis (0.8%) and DNA blotting on Biodyne membrane (Pall) were performed as described by Kohiyama *et al.* (1986). Hybridization with [^{32}P]-labelled pHV2 DNA was carried out for 16 h at 42 °C in a buffer containing 50% (v/v) formamide; 5× SSC; 1× Denhardt's solution (Maniatis *et al.*, 1982); 20 mM-sodium phosphate (pH 6.5); 250 µg denatured *E. coli* DNA ml⁻¹ and 10% (w/v) dextran sulphate. Filters were washed first at room temperature in 2× SSC, 0.2% SDS, then at 65 °C in 0.1× SSC, 0.1% SDS.

Results

Continuous DNA synthesis in minimal medium

The generation time in a *his-1* mutant of *Haloferax volcanii* is 6–7 h when grown in minimal medium supplemented with histidine (MM⁺), with continuous agitation at 37 °C. When histidine is not present in the minimal medium (MM⁻), growth decreases rapidly and

ceases completely after about one doubling time. Amino acid starvation is sufficient to block protein synthesis with no significant effect on viability during the lag time in MM⁻ (data not shown). After the addition of histidine, bacterial growth and protein synthesis resume (Fig. 1a). DNA synthesis was measured in MM⁺ and MM⁻ by fluorometry (see Methods). Fig. 1b shows that the quantity of total DNA per cell increases continuously for at least 50 h, despite the absence of concomitant protein synthesis. The rate of increase in DNA is comparable to about 15% of that observed during exponential growth in MM⁺, and the DNA content in MM⁻ increases 2.5-fold in approximately 50 h.

DNA synthesis is not due to residual protein synthesis

Anisomycin, a specific inhibitor of protein synthesis in eukaryotes, has also been found to inhibit synthesis in some archaeobacteria (Bonelo *et al.*, 1984). The minimal inhibitory concentration (MIC) of anisomycin for *H. volcanii* was determined to be 10 µg ml⁻¹ in minimal medium (data not shown). At this concentration, the rate of protein synthesis is reduced by 93%, as shown in the inset of Fig. 2 by following the radioactive labelling of proteins. In order to eliminate the possibility that continuous DNA synthesis results from residual protein synthesis during amino acid starvation, the effect of inhibiting translation by anisomycin in the *his-1* mutant was examined. When the *his-1* mutant was grown in MM⁺ containing anisomycin (10 µg ml⁻¹), continuous DNA synthesis occurred comparable to that in the absence of histidine (MM⁻). In addition, this increase in DNA concentration was also observed even in MM⁻ containing anisomycin (Fig. 2). These results show that the residual continuing DNA synthesis is independent of concomitant protein synthesis.

Continuous DNA synthesis in complete medium (CM)

To verify that the increase in DNA concentration during inhibition of translation is independent of the type of medium, DNA synthesis was studied in complete medium in the presence of 10 µg anisomycin ml⁻¹. *H. volcanii* has a generation time of 5 h when grown in complete medium without anisomycin (CM⁻) at 37 °C. When anisomycin (10 µg ml⁻¹) is added to the medium, protein synthesis ceases quickly, as shown by measuring L-[^3H]alanine incorporation (Fig. 3a). As expected, this is immediately followed by a drastic decrease in the growth rate and cell division ceases completely in 12–15 h. Plating dilutions of culture samples on MM⁺ agar plates confirmed the lack of difference in number of viable cells during the lag time in MM⁻. In parallel, DNA synthesis was measured by [^3H]thymidine incorporation. After

Fig. 2. Effect of anisomycin and histidine deficiency on DNA replication in a *his-1* mutant of *H. volcanii*. The inset shows the effect of anisomycin on protein synthesis in MM⁺. Incorporation of L-[^3H]alanine in anisomycin-treated cells is expressed as a percentage of that in control cells (without anisomycin) 5 h after the beginning of the treatment. The total DNA per 300 µl of bacterial culture was measured at different times using DAPI determination. (▲) Exponential growth without anisomycin; (▼) without amino acid starvation and with anisomycin (10 µg ml⁻¹); (■) amino acid starvation without anisomycin; (●) amino acid starvation with anisomycin (10 µg ml⁻¹).

Fig. 3. Effect of anisomycin on bacterial growth, DNA synthesis and protein synthesis in the *his-1* mutant of *H. volcanii*. An exponential culture of the *his-1* mutant in complete medium was divided into four portions. Anisomycin ($10 \mu\text{g ml}^{-1}$) was added to two portions at 0 h (●, ■). The cultures without anisomycin (▲, ▼) served as control. (a) For measurement of protein synthesis $1.4 \mu\text{Ci L-}^3\text{H}$ alanine ml^{-1} was added to both types of culture at 0 h and incubation was continued for the indicated times at 37°C . (▲) Culture without anisomycin, OD₆₀₀ (log scale); (▼) culture without anisomycin, radioactive labelling (linear scale); (●) culture with anisomycin, OD₆₀₀ (log scale); (■) culture with anisomycin, radioactive labelling (linear scale). (b) For DNA synthesis measurement $1 \mu\text{Ci } ^3\text{H}$ thymidine ml^{-1} was added to both types of culture and DNA synthesis was measured by radioactive labelling of bacteria. (▼) Culture without anisomycin; (■) culture with anisomycin.

gradual slow increase of DNA synthesis, the linear incorporation of thymidine was found to continue for as long as 75 h in the presence of anisomycin (Fig. 3b).

Continuous DNA synthesis is sensitive to aphidicolin

We have previously shown that DNA replication in halophilic archaeobacteria is inhibited by aphidicolin (Forterre *et al.*, 1984). DNA synthesis in the *H. volcanii* mutant was measured by ^3H thymidine labelling in the presence of various concentrations of aphidicolin. At $20 \mu\text{g ml}^{-1}$, DNA synthesis was inhibited by 95% (inset to Fig. 4).

We have looked for the effect of aphidicolin on the DNA synthesis observed in the absence of protein synthesis. As shown in Fig. 4, ^3H thymidine incorporation into *his-1* mutant DNA is inhibited by aphidicolin ($20 \mu\text{g ml}^{-1}$) in both MM⁻ and MM⁺ bacterial cultures. This result shows that this continuous DNA synthesis, like normal DNA replication, is sensitive to aphidicolin. A similar result was obtained in rich medium in the presence of anisomycin (data not shown).

Continuous DNA synthesis concerns chromosomal but not pHV2 DNA

The *his-1* mutant was grown in MM⁻ or MM⁺ containing radioactive precursors of DNA synthesis ($2 \mu\text{Ci } [\alpha\text{-}^{32}\text{P}]\text{dTTP ml}^{-1}$ or $0.5 \mu\text{Ci } [^{14}\text{C}]\text{thymidine ml}^{-1}$). Total

^{32}P -labelled DNA was then extracted and subjected to agarose gel electrophoresis. Autoradiography of the dried gel (Fig. 5a) showed that under normal conditions of bacterial growth both chromosomal and pHV2 (6.4 kb) plasmid DNAs were labelled (lane 2), whereas in amino acid-starved bacteria only the chromosomal DNA was labelled (lane 1). Thus, chromosomal DNA but not pHV2 plasmid DNA undergoes continued replication in the absence of protein synthesis. When these DNA samples were digested with the restriction endonucleases *Pst*I (Fig. 5b part A) or *Xho*I (Fig. 5b part B), the pattern of labelled fragments from amino-acid-starved bacteria was generally similar to that of bacteria grown in the presence of histidine, except for some specific fragments (6.4 kb *Pst*I–*Pst*I, 1.84 kb *Xho*I–*Xho*I, 1.25 kb *Xho*I–*Xho*I, 1.22 kb *Xho*I–*Xho*I, 0.99 kb *Xho*I–*Xho*I), which were preferentially labelled when the culture was not subjected to amino acid starvation (Fig. 5b, lanes 2). The sizes of these restriction fragments of pHV2 DNA corresponded to those observed for the pHV2 DNA digestion pattern (Charlebois *et al.*, 1987). This suggests that the entire chromosomal DNA is replicated during continuous DNA synthesis. Thus, continuous synthesis involves the entire chromosomal DNA but not the pHV2 plasmid.

To confirm these results, total DNA from normal and anisomycin-treated bacteria was hybridized with a pHV2 ^{32}P DNA probe (Fig. 6). In Fig. 6c, we show the densitometric analysis of the pHV2 plasmid band

Fig. 4. Effect of aphidicolin and histidine deficiency on DNA replication in a *his-1* mutant of *H. volcanii*. The inset shows the effect of aphidicolin on DNA synthesis during exponential growth in minimal medium supplemented with histidine (MM^+). Incorporation of [^3H]thymidine in aphidicolin-treated cells is expressed as a percentage of that in control cells (without aphidicolin) 15 h after the beginning of the treatment. An exponential culture of the *his-1* mutant in MM^+ medium was washed with minimal medium without histidine (MM^-) and divided into 2 portions. Histidine was added to one at 0 h and this culture served as a control. [^3H]Thymidine ($1 \mu\text{Ci ml}^{-1}$) was then added to both cultures at 0 h and DNA synthesis was measured by radioactive labelling of bacteria. Aphidicolin ($20 \mu\text{g ml}^{-1}$) was also added at this time. ($\blacktriangle$) Without aphidicolin during exponential growth; ($\blacktriangledown$) without aphidicolin in amino-acid-starved bacteria; ($\bullet$) with aphidicolin and without amino acid starvation; ($\blacksquare$) with aphidicolin in amino-acid-starved bacteria.

intensities on Southern blots (Fig. 6b). It can be seen that the quantity of supercoiled pHV2 plasmid (arrow) is higher in total DNA from untreated than from anisomycin-treated bacteria. This is in agreement with the hypothesis that the pHV2 plasmid DNA does not replicate during the continuous DNA synthesis. The inhibition of pHV2 plasmid replication is reversible, since plasmid DNA synthesis resumes after the removal of anisomycin from the culture in complete medium or after the addition of histidine to bacterial cultures in MM^- (data not shown). The upper band mostly corresponds to the position of chromosomal DNA. The fact that it is recognized by the pHV2 [^{32}P]DNA probe might be due to the presence of some high-molecular-mass forms of the plasmid since we have not noticed any

unspecific hybridization using the pHV2 [^{32}P]DNA probe.

Discussion

We have presented evidence that *H. volcanii* continues to replicate its DNA for up to 75 h during inhibition of protein synthesis with an apparent biphasic rate synthesis the first part of which is probably the run out of normal replication forks. As the quantity of DNA in *H. volcanii* increases 2.5-fold in about 50 h as shown using fluorometric DNA estimation (Fig. 1b, Fig. 2), reinitiation might occur in the absence of concomitant protein synthesis. We were unsuccessful in our attempt to use density labelling of bacteria, probably due to poor incorporation of 5-bromodeoxyuridine in *H. volcanii* (whose DNA is GC-rich). So, the possibility of reinitiation remains to be demonstrated since we have no information about the length of the C and D replication periods in the archaeobacteria kingdom.

Analysis of DNA replication patterns shows all the DNA of *H. volcanii* to be replicated in minimal medium without histidine with the exception of the pHV2 plasmid DNA. This is unlike some bacterial plasmids, such as the *E. coli* colicinogenic factor E1 (ColE1), where DNA replication is initiated during amino acid starvation (Bazara & Helinski, 1970).

The continuous DNA synthesis cannot be explained by residual run out or by repair-like synthesis since: (i) repair-like DNA synthesis does not normally result in an increase in the quantity of DNA. Thus, the increase in the DNA quantity shown with the DAPI method excludes this possibility. (ii) Increase (2.5-fold) of the quantity of DNA might suggest that reinitiation may occur during inhibition of protein synthesis. In the case of 'dichotomous' replication, as seen in *E. coli*, additional rounds of replication must, therefore, start on the same chromosome before previous rounds have terminated (McMacken *et al.*, 1987). Hence, run out of replication forks might result in a bigger increase in the quantity of DNA. However, this kind of phenomenon usually happens when cells grow with a short doubling time or in minimal medium. (iii) This DNA synthesis is sensitive to aphidicolin, suggesting that it uses an aphidicolin-sensitive DNA polymerase as is the case with normal DNA replication. This kind of DNA polymerase has been purified from another halophilic archaeobacterium, *Halobacterium halobium* (Nakayama & Kohiyama, 1985).

Under certain circumstances, *E. coli* exhibits altered modes of DNA replication. Induced stable DNA replication can occur in the absence of concomitant protein synthesis but only if DNA replication has been previously inhibited as during thymine starvation in a

Fig. 5. DNA replication pattern during amino acid starvation of the *his-1* mutant. An exponential culture of *H. volcanii* in minimal medium supplemented with histidine (MM^+) was washed with the same medium without histidine (MM^-) and divided into two portions. Histidine was added to one, which served as control. Two radioactive pulses were done, one in the exponential culture in MM^+ medium (lanes 2) and the second in the amino-acid-starved bacteria in MM^- medium (lanes 1). Samples were taken and the total DNA was extracted, digested or not, the same quantity of radioactivity was electrophoresed and autoradiographed. The arrowhead indicates the position of pHV2 plasmid DNA. (a) $[^{32}\text{P}]\text{dTTP}$ pulses of 1 h after 45 h of amino acid starvation in the culture without anisomycin, and during exponential growth for the culture with histidine. The total DNA was applied to an agarose gel without prior digestion. (b) $[^{14}\text{C}]\text{thymidine}$ pulses for $0.5 \times$ doubling time in the exponentially-growing culture and for 19 h in the amino-acid-starved culture. (A) *Pst*I digestion of the DNA samples. (B) *Xho*I digestion. The molecular mass markers are indicated on the right.

thymine-dependent mutant (Kogoma & Lark, 1975; Magee & Kogoma, 1990). Mutants constitutive for stable DNA replication, i.e. not requiring induction, have been isolated and found to be *rnh* mutants, lacking RNAase H (Kogoma *et al.*, 1985; Kogoma, 1986). In this respect, DNA synthesis in the absence of protein synthesis in *H. volcanii* resembles the latter. There are, however, some important differences. First, we do not know at this point whether the observed residual DNA replication is a normal fraction of total DNA synthesis in *H. volcanii* or whether it is induced by the treatments which stop protein synthesis. Second, the total quantity of DNA synthesized after 60 h in *H. volcanii* is considerably less than that observed in a *rnh* mutant of *E. coli* (Kogoma, 1986).

Further experiments will be necessary to determine whether this particular type of DNA synthesis is part of the normal replication in *H. volcanii* and what importance it can play in replication. On the other hand, our data might indicate that during the inhibition of protein synthesis, a change occurs in the cell which results in the ability to initiate cycles of replication in the absence of further protein synthesis. This potential should be stable and might involve a mechanism which is re-used in several cycles of DNA replication, as in RNAase H-defective mutants of *E. coli*. The proteins involved would be part of the normal replication apparatus, since aphidicolin, which affects normal DNA replication, also affects the continuous DNA replication observed after the inhibition of protein synthesis.

The production of supercoiled pHV2 plasmid is stopped during the inhibition of protein synthesis by anisomycin or by amino acid starvation. The DNA replication pattern of amino-acid-starved bacteria shows that the pHV2 plasmid is not labelled and that its replication is stopped (Fig. 5). The initiation of pHV2 replication might require labile factors which must be synthesized in each cell cycle as described for most *E. coli* plasmids.

The inhibition of supercoiled pHV2 monomer DNA production is confirmed by hybridization experiments, but the upper signal in Fig. 6 reveals the existence of some kind of high-molecular-mass plasmid multimers.

Some of our preliminary results (data not shown) suggest that the upper signal in Fig. 6 might be due to

Fig. 6. pHV2 plasmid DNA does not replicate during inhibition of protein synthesis in complete medium. An exponential culture of the *his-1* mutant in CM without anisomycin (CM^-) was split into two portions. To one (CM^+) $10\ \mu\text{g}$ anisomycin ml^{-1} were added (lanes 1). The portion without anisomycin served as control (lanes 2). A sample of the anisomycin-treated culture was taken after 45 h. Equal amounts of total cell DNA were subjected to 0.8% agarose gel electrophoresis in the presence of ethidium bromide (a). The gel was transferred onto a Biotrans membrane and hybridized with a [^{32}P]pHV2 probe (b). (c) Densitometric scan of (b). The arrowhead indicates the position of the supercoiled pHV2 monomer DNA. sr: Surface ratio.

some concatemeric forms of plasmid or to unresolved pHV2 plasmid replication intermediates which might be resolved only in the presence of protein synthesis, thus

allowing production of supercoiled pHV2 monomer DNA.

Further experiments are necessary to elucidate the mechanism of pHV2 plasmid replication and the effect of protein synthesis on this mechanism.

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