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SOS Induction in *Escherichia coli* by Infection with Mutant Filamentous Phage That Are Defective in Initiation of Complementary-Strand DNA Synthesis

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We report that the SOS response is induced in *Escherichia coli* by infection with mutant filamentous phage that are defective in initiation of the complementary (minus)-strand synthesis. One such mutant, R377, which lacks the entire region of the minus-strand origin, failed to synthesize any detectable amount of primer RNA for minus-strand synthesis. In addition, the rate of conversion of parental single-stranded DNA of the mutant to the double-stranded replicative form in infected cells was extremely slow. Upon infection, R377 induced the SOS response in the cell, whereas the wild-type phage did not. The SOS induction was monitored by (i) induction of β-galactosidase in a strain carrying a *dinD*::*lacZ* fusion and (ii) increased levels of RecA protein. In addition, cells infected with R377 formed filaments. Another deletion mutant of the minus-strand origin, M13ΔE101 (M. H. Kim, J. C. Hines, and D. S. Ray, Proc. Natl. Acad. Sci. USA 78:6784–6788, 1981), also induced the SOS response in *E. coli*. M13Gori101 (D. S. Ray, J. C. Hines, M. H. Kim, R. Imber, and N. Nomura, Gene 18:231–238, 1982), which is a derivative of M13ΔE101 carrying the primase-dependent minus-strand origin of phage G4, did not induce the SOS response. These observations indicate that single-stranded DNA by itself induces the SOS response in vivo.

DNA damage in *Escherichia coli* results in induction of the SOS genes, which include many genes involved in DNA repair. The SOS induction is due to cleavage of the LexA protein, the repressor of the SOS genes, by the action of activated RecA protein. Damage to DNA leads to production of an inducing signal, which in turn activates the RecA protein (for a review, see reference 39). The exact nature of the in vivo signal for the SOS induction has been controversial. In vitro studies have revealed that RecA can be activated by forming a ternary complex with single-stranded DNA and a nucleoside triphosphate (5, 6). However, there has been no direct in vivo evidence that single-stranded DNA by itself can activate the RecA protein and induce the SOS functions. In this work, we have taken advantage of the properties of mutants of a filamentous bacteriophage to provide a source of single-stranded DNA, allowing us to test this model directly.

The genome of filamentous bacteriophages consists of a circular single-stranded DNA (6.4 kb). Upon infection of *E. coli* cells, the viral single-stranded DNA is converted to the double-stranded replicative form (RF) by synthesis of the complementary (minus) strand. The parental RF thus produced serves as a template for the synthesis of more RF molecules during the early stage of infection (the first 10 or 15 min). No single-stranded DNA accumulates during this stage. In the later stage of infection (starting from about 10 to 15 min after infection), asymmetric synthesis of single-stranded viral (plus-strand) DNA occurs on the accumulated RF molecules by a rolling-circle mechanism. The viral single strands produced are bound by the phage gene V protein, accumulate in the cell in the form of the complex, and are subsequently packaged into viral particles (for reviews, see references 20, 28, 42).

The synthesis of minus strands starts from an RNA primer, which is synthesized by the action of host RNA polymerase at a unique site within the intergenic region of the phage chromosome (the minus-strand origin) (8). Mutant phages that are defective in the function of the minus-strand origin can be obtained. These mutants grow slowly and form tiny turbid plaques. Presumably, the mutants can grow by using a secondary, yet uncharacterized, inefficient mechanism to synthesize the minus strand (19).

In this paper, we report that the SOS functions are induced in *E. coli* by infection with mutant filamentous phage that are defective in the minus-strand origin. We propose that single-stranded DNA itself is the SOS-inducing signal in vivo.

MATERIALS AND METHODS

Bacteria, bacteriophage, and media. *E. coli* K38 [HfrC (λ) *phoA6 tonA22 garB10 ompF627 relA1 pit-10 spoT1* $T2^r$ *PO2A*] (15) was used in general. JH137 [K38 Δ*lacZ dinD1*::Mu dI (Ap *lac*)] (12), obtained from J. Heitman, was used to monitor the SOS induction by β-galactosidase activity. AB1899 (*lon-1 thr-1 leuB6 thi-1 argE3 his-4 proA2 lacY1 galK2 mtl-1 xyl-5 ara-14 rpsL31 tsx-3 supE44*) (17) and GC2597 (*sfiA*::Tn*5 pyrD thr leu his lac gal malB srl*::Tn*10 sfiC str*) were obtained from the Stock Center at the National Institute of Genetics, Japan. NH103 (AB1899F^+::Tn*10*) was constructed by crossing AB1899 with K866, which was obtained from M. Russel. NH104 was obtained by transducing K38 to *sfiA*::Tn*5* with P1 grown in GC2597. Filamentous bacteriophage f1 was described previously (14, 28). R377, a mutant phage carrying a deletion ranging from nucleotides 5617 to 5766, was constructed as follows. Start-

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ing from R252 (29), carrying an *Eco*RI site at position 5616, R368 was constructed; in addition to the *Eco*RI site, R368 carries a unique *Pst*I site at position 5766. R377 was obtained by cleavage of R368 RF with *Eco*RI and *Pst*I and ligation after the ends were made blunt with the Klenow fragment of DNA polymerase I. The exact extent of the deletion in R377 was confirmed by nucleotide sequencing by the dideoxynucleotide method (35). M13ΔE101 (19) and M13Gori101 (31) were obtained from D. S. Ray. Bacteria and phage were propagated in tryptone medium (41).

Enzymes and chemicals. Restriction endonucleases, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England BioLabs. *E. coli* RNA polymerase holoenzyme and single strand binding protein (SSB) were generously provided by A. Ishihama and N. Shimamoto, respectively, of the National Institute of Genetics. Purified RecA protein and anti-RecA antibody were kindly provided by T. Ogawa of Osaka University and K. Tamai of the Medical Biological Laboratories, respectively. $^{32}P_i$ and [α-^{32}P]GTP (800 Ci/mmol) were purchased from DuPont/NEN Research Products. Nonradioactive nucleoside triphosphates were from Boehringer-Mannheim. Bovine serum albumin was from Takara Shuzo Co. Mitomycin C was from Sigma.

In vitro primer RNA synthesis. Primer RNA synthesis was carried out in a 20-μl reaction mixture containing 0.1 pmol of phage DNA, 120 pmol (2.4 μg) of SSB, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–KOH (pH 7.5), 130 mM KCl, 0.02 mg of bovine serum albumin per ml, 5 mM $MgCl_2$, 500 μM ATP, CTP, and TTP, 50 μM GTP, 10 μCi of [α-^{32}P]GTP (800 Ci/mmol), and 1 pmol of RNA polymerase holoenzyme. Reactions were carried out at 30°C for 20 min and stopped by the addition of 80 μl of a stop solution that gave final concentrations of 80 mM NaCl and 10 mM EDTA. The nucleic acids were precipitated with 250 μl of ethanol, dried, and suspended in 10 μl of blue mix (80% formamide containing bromophenol blue and xylene cyanol). The samples were boiled for 2 min, put on ice, and subjected to electrophoresis through 12% polyacrylamide–8 M urea gels in 0.5× TBE buffer (34) at 300 V until the bromophenol blue reached near the bottom. The running buffer was 0.5× TBE. After electrophoresis the gel was soaked in 5% acetic acid–5% methanol for 10 min and then in 5% methanol for 10 min, dried, and exposed to Fuji X-ray film.

^{32}P-labeled phage. ^{32}P-labeled phage were prepared essentially as described by Kim et al. (19). K38 cells were grown to 2 × 10^8 per ml at 37°C in 1 ml (for wild-type f1) or 3 ml (for mutant phage R377) of medium (0.1 M Tris-HCl [pH 7.0], 2.5 mM Na_2SO_4, 37 mM NH_4Cl, 27 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM Na_2HPO_4, 0.5% glucose, 2 μg of thymidine per ml, and 20 L-amino acids, each at 50 μg/ml) (30) and infected with phage at a multiplicity of 10 in the presence of 0.1 mCi of $^{32}P_i$ per ml. After 4.5 h (for the wild type) or 5.5 h (for R377) of growth, the culture was centrifuged, and the phage in the supernatant were precipitated with polyethylene glycol and suspended in tryptone medium.

Determination of parental RF in cells infected with ^{32}P-labeled phage. *E. coli* K38 cells were grown in tryptone medium. At a density of 1 × 10^8 to 2 × 10^8 cells per ml, the cells were infected with ^{32}P-labeled phage at a multiplicity of infection of 10. Chloramphenicol was added to 100 μg/ml 20 min before infection. Aliquots (1 ml) of each culture were harvested at 15 min (wild type, R377) and 45 min (R377) after infection and washed twice with saline. The cells were lysed with 100 μl of lysozyme (5 mg/ml in 10% sucrose–50 mM Tris-HCl [pH 8.0]) and 100 μl of 0.2 M EDTA for 10 min on ice, and then 100 μl of 5% sodium dodecyl sulfate (SDS)–7.5 M ammonium acetate was added. After centrifugation the supernatant was saved, and the nucleic acid was precipitated with ethanol and treated with RNase. The samples were subjected to electrophoresis through a 1% agarose gel at 80 V. The gel was dried and exposed to Fuji X-ray film.

Western immunoblotting. Cells were boiled for 5 min in 2× SDS–loading buffer (63 mM Tris-HCl [pH 6.8], 4% SDS, 5% β-mercaptoethanol, 20% glycerol), and slab gel electrophoresis was performed with a discontinuous 12% polyacrylamide gel system containing SDS as described by Laemmli (21). Electrotransfer of protein to the nitrocellulose membrane and immunochemical detection of the antigen were carried out as described previously (33) using anti-RecA antibody and anti-immunoglobulin coupled to horseradish peroxidase.

Extraction and gel electrophoresis of phage DNA in infected cells. K38 cells were grown in tryptone medium. At a density of 2 × 10^8 cells per ml, the cells were infected with either wild-type f1 or R377 at a multiplicity of 20. Aliquots (1 ml) of each culture were harvested at various times after infection. Cells were precipitated, washed, suspended in 100 μl of an ice-cold solution of 10% sucrose–50 mM Tris-HCl (pH 8.0), and lysed with 100 μl of a lysozyme solution (20 mg/ml in 0.25 M Tris-HCl [pH 8.0]) and 80 μl of 0.25 M EDTA for 10 min. Then 40 μl of 10% SDS–5 M NaCl was added, and after incubation overnight at 4°C the supernatant was collected, extracted with phenol-chloroform (1:1), precipitated with ethanol, dried, and treated with RNase. The DNA was subjected to electrophoresis at 80 V through a 0.7% agarose gel containing ethidium bromide (0.5 μg/ml).

RESULTS

Defective minus-strand origin of R377. Mutant phage R377, which is defective in the origin function for the minus strand synthesis, was constructed by deleting a region of the genome from nucleotides 5616 to 5767 (see Materials and Methods). The missing region contains two large hairpin turns (Fig. 1) that are involved in the initiation of minus-

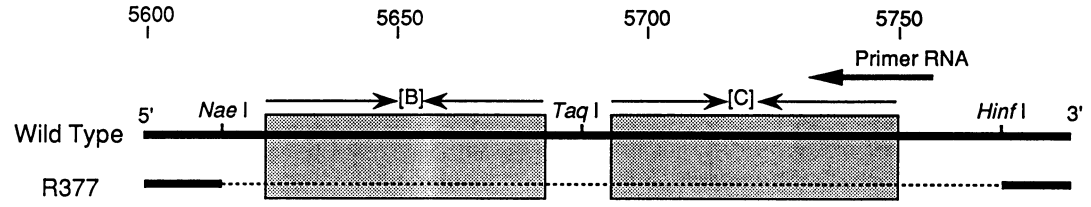

FIG. 1. Map of the minus-strand origin region of phage f1. Shaded boxes show the regions of potential hairpin structure (1). The dotted line indicates the sequence deleted in the mutant R377. The thick arrow represents the RNA primer for the minus-strand synthesis (8). The numerals on the top indicate nucleotide numbers.

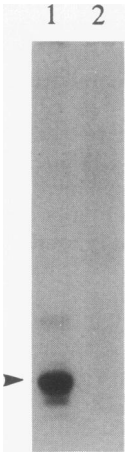

FIG. 2. Primer RNA synthesis in vitro carried out as described in Materials and Methods with single-stranded viral DNA of wild-type f1 or R377 as the template. The reaction product was electrophoresed in a 12% polyacrylamide–8 M urea gel, which was then dried and radioautographed. Lanes: 1, wild-type f1 single-stranded DNA; 2, R377 single-stranded DNA. The arrowhead indicates the primer RNA.

strand synthesis (11, 13, 37). R377 forms very small and turbid plaques, and its growth rate (progeny phage yield per infected cell) is approximately 1/100 of that of the wild-type f1 phage (data not shown).

As an assay for function of the minus-strand origin, we carried out an in vitro reaction of primer RNA synthesis, which consisted of viral single-stranded DNA, *E. coli* RNA polymerase, and *E. coli* SSB (8). The results shown in Fig. 2 indicate that R377 DNA did not yield any detectable amount of primer RNA, whereas the wild-type DNA produced a primer RNA about 20 nucleotides long.

In addition, we measured DNA synthesis on a viral single-stranded DNA template in a crude extract of *E. coli* by using a system described by Wickner et al. (40). The result showed that single-stranded DNA of R377, unlike that of wild-type f1, was inactive as a template for DNA synthesis (data not shown).

Another assay we used was in vivo conversion of ^{32}P-labeled phage single-stranded DNA to the double-stranded RF upon infection. Whereas the wild-type viral DNA was quickly converted to RFI and RFII after infection, the conversion of R377 phage DNA to the RF was extremely slow and inefficient (Fig. 3). These results indicate that the initiation step of minus-strand synthesis, which converts single-stranded plus-strand DNA to the double-stranded RF, is defective in R377.

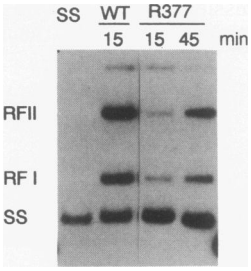

FIG. 3. Parental RF formation in phage-infected cells. K38 cells were infected with ^{32}P-labeled wild-type f1 or R377 in the presence of chloramphenicol. At 15 or 45 min after infection (as indicated), intracellular DNA was extracted, electrophoresed, and radioautographed as described in Materials and Methods.

SOS induction in cells infected with R377. We tested whether SOS induction occurred in *E. coli* cells upon infection with R377 by using a tester strain JH137 (12), which carries a *lacZ* gene under the control of a promoter of an SOS-responsive gene, *dinD* (18). The activity of β-galactosidase in this strain increased to a level fivefold higher than the background after infection with R377, whereas the enzyme activity remained at the background level in cells infected with the wild-type phage (Fig. 4). Thus, R377 induces the SOS functions upon infection, whereas the wild-type f1 phage does not.

To further confirm this point, we used anti-RecA antibodies to measure the level of RecA protein in extracts of phage-infected cells. The results of Western blotting experiments (Fig. 5) indicate that R377, but not the wild-type phage, induced synthesis of the 40-kDa RecA protein.

The specific function of RecA in the SOS system was initially deduced from studies of lambda phage induction (32). Activated RecA mediates proteolytic cleavage of the lambda repressor, which in turn induces growth of lambda phage in lysogenic bacteria. We found that the titer of lambda phage in the culture of a lysogen increased after infection with R377 but not with wild-type f1 (data not shown).

It is known that *E. coli* cells exposed to SOS-inducing treatments continue to divide but are inhibited in septation by the action of induced *sfiA* (*sulA*) gene and thus form filaments (39). Figure 6 shows the morphology of phage-infected cells of three *E. coli* strains: K38 (wild type), NH103 (*lon*), and NH104 (*sfiA*). In all three strains, cells infected with the wild-type f1 phage (Fig. 6d, e, and f) were indistinguishable from uninfected cells (Fig. 6a, b, and c). On the other hand, K38 and NH103 cells infected with R377 formed filaments (Fig. 6g and h), whereas R377-infected NH104 cells

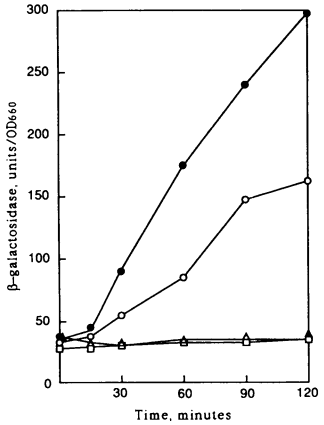

FIG. 4. Induction of β-galactosidase by phage infection of *dinD*::Mud(Ap *lac*) fusion strain JH137. Cells were grown in tryptone medium at 37°C to a cell density of 1×10^8 to 2×10^8 cells per ml and then either infected with phage (multiplicity of infection, 10) or mixed with a solution of mitomycin C (final concentration, 0.25 μg/ml) and further incubated at 34°C. Aliquots (0.1 ml) were removed at various times, and β-galactosidase activity was determined as described previously (27). Specific activity is given in terms of units of enzyme per A_{660} unit of the culture. Symbols: □, uninfected cells; △, cells infected with wild-type f1; ○, cells infected with R377; ●, cells treated with mitomycin C (0.25 μg/ml).

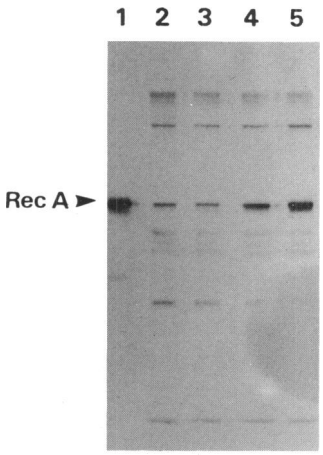

FIG. 5. Induction of RecA protein by phage infection. Exponentially growing cells (JH137) were infected with phage or treated with mitomycin C as described in the legend to Fig. 4. After 2 h of incubation at 34°C, cells (0.004 A_{660} unit) were boiled for 5 min in a loading buffer and then subjected to electrophoresis and Western blotting with anti-RecA antibody as described in Materials and Methods. Lanes: 1, purified RecA protein (20 ng); 2, uninfected cells; 3, cells infected with wild-type f1; 4, cells infected with R377; 5, cells treated with mitomycin C (0.25 μg/ml). On lane 1, purified RecA protein (20 ng) was electrophoresed.

did not (Fig. 6i). These results indicate that the filamentation observed in R377-infected cells is due to the SOS induction. Filamentation of K38 cells infected with R377, unlike that of NH103 cells, was not very remarkable and was only observed in about 30% of the cell population (Fig. 6g), in spite of the fact that more than 80% of the cells were infected (data not shown). This observation may reflect transience of the SOS signal produced by R377 infection.

SOS induction by another minus-strand origin mutant. M13ΔE101, another deletion mutant of the minus-strand origin, was isolated by Kim et al. (19). They also constructed M13Gori101, in which the minus-strand origin of G4, another single-stranded DNA phage, was inserted into M13ΔE101 (31). Infection with M13ΔE101 resulted in the SOS induction, whereas infection with M13Gori101 did not (Table 1).

Accumulation of phage DNA in infected cells. Intracellular levels of phage RF and single-stranded DNA in R377-infected and f1-infected cells were compared (Fig. 7). Although no single-stranded DNA was observed intracellularly at 10 min after infection with wild-type f1, a small amount of single-stranded DNA was detected at 10 min after R377 infection. This single-stranded DNA, which was estimated to be present at approximately two or three molecules per cell by quantification of the band, represents the parental R377 DNA and appears to be responsible for the SOS induction in the cells. On the other hand, in the f1-infected cells the viral DNA was completely converted to the RF at this time point.

In later stages of infection (20 and 40 min), the wild-type f1 produced much larger amounts of RF and single-stranded DNA than R377 did (Fig. 7). The RF and single-stranded DNA of R377 produced in the late stages must be due to a secondary mechanism of minus-strand synthesis.

DISCUSSION

When DNA in *E. coli* cells is damaged, an inducing signal that activates RecA protein is generated. The activated RecA cleaves and thus inactivates LexA protein, the repressor of the SOS genes, leading to induction of the SOS response. In vitro, RecA is activated when it forms a complex with single-stranded DNA and a nucleoside triphosphate (5, 6). However, the exact nature of the inducing signal in vivo has been controversial, reflecting the wide variety of treatments that lead to the SOS response. Smith and Oishi (38) proposed that different types of DNA damage lead to degradation of DNA by specific DNases, which produce the inducing signal. Lu and Echols (22) proposed that interaction of RecA with pyrimidine dimers in double-stranded DNA leads to the LexA cleavage reaction.

In the present study we demonstrated that the SOS response was induced by infection of *E. coli* with R377, a mutant filamentous phage that is defective in initiation of the complementary-strand synthesis, the reaction which converts the single-stranded viral DNA to double-stranded DNA (Fig. 4). Infection with M13ΔE101 (19), a similar mutant phage, also induced the SOS response. Furthermore, no SOS induction was detected upon infection with M13Gori101 (31), a derivative of M13ΔE101 that carries a different system to convert single-stranded DNA to the RF: the primase (DnaG)-dependent minus-strand origin of phage G4 (Table 1). These observations indicate that single-stranded DNA by itself induces the SOS functions in vivo, as has been suggested from in vitro studies (5). Since R377 single-stranded DNA does not serve as a template for synthesis of the primer RNA, the single-stranded DNA without any replication fork is likely to induce the SOS response.

Although there has been no direct in vivo evidence that single-stranded DNA by itself can induce the SOS functions, it has been shown that the SOS induction by UV light is blocked when DNA replication is inhibited (33, 36). Sassanfar and Roberts (36) interpreted this to mean that DNA replication produces gaps at damage-induced lesions and thus provides the single-stranded DNA that activates RecA protein. Our results reported here support this model. The observation that SOS induction by nalidixic acid requires the DNA-unwinding activity of the RecBCD enzyme (4) is also in accordance with this notion. Furthermore, D'Ari and Huisman (7) reported that infection of *E. coli* with UV-irradiated phage P1, λ, or M13 induced the SOS response. Since formation of the double-stranded RF of a single-stranded DNA phage is disturbed by UV irradiation of the phage (2), SOS induction by irradiated M13 may be due to the single-stranded parental phage DNA.

DNA replication of filamentous phage occurs in three steps (20). (i) Upon infection, the viral single-stranded DNA (plus strand) is converted to the double-stranded RF by the synthesis of minus-strand DNA. No more than about three DNA molecules enter the cell, even at high multiplicities, as though only one phage may enter per F pilus (23). (ii) The RF molecule thus produced replicates, during the early stage of infection, to form a pool of RF molecules. The plus strands are synthesized on RF molecules by a rolling-circle mechanism (9), in which a newly synthesized plus strand displaces the old plus strand of the RF, resulting in the formation of a double-stranded RF molecule and a single-stranded plus-strand circle upon completion of a round of synthesis. On the single-stranded circle thus produced, the minus strand is quickly synthesized in the same way as in the formation of parental RF (16). (iii) In the later stage of infection, when the phage gene V protein accumulates, it binds to the single-stranded DNA and prevents it from being used as template for the minus-strand synthesis (24, 25). This results in

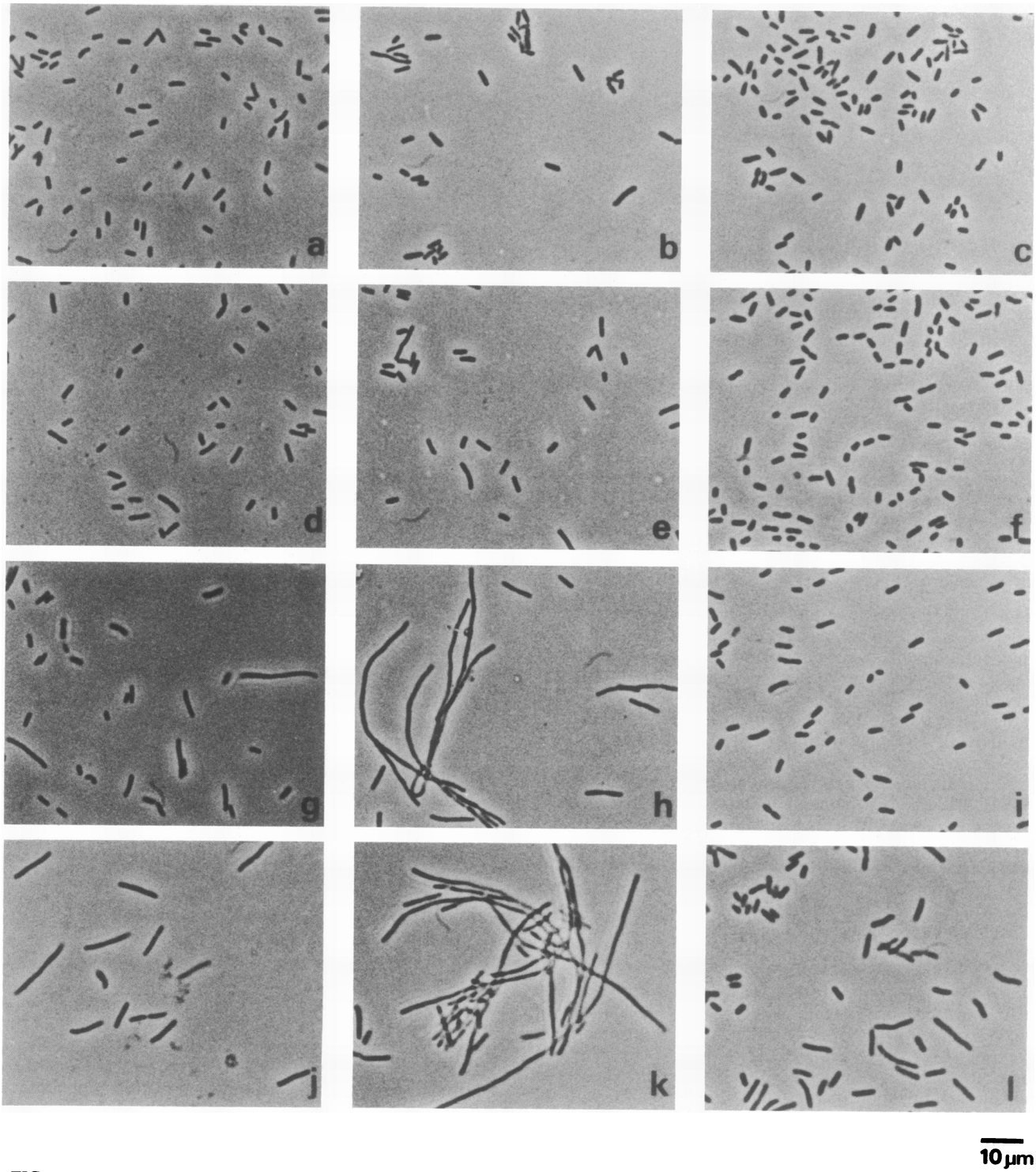

FIG. 6. Morphology of phage-infected cells. Exponentially growing cultures of three *E. coli* strains, K38 (wild type) (a, d, g, and j), NH103 (*lon*) (b, e, h, and k), and NH104 (*sfiA*) (c, f, i, and l), were infected with phage at a multiplicity of infection of 30 or treated with mitomycin C (0.25 μg/ml). After 2 h of incubation at 34°C the cells were photographed under microscope. (a, b, and c) Uninfected cells; (d, e, and f) cells infected with wild-type f1; (g, h, and i) cells infected with R377; (j, k, and l) cells treated with mitomycin C.

asymmetric synthesis and accumulation of the plus strand in single-stranded form.

Synthesis of minus-strand DNA of filamentous phage is initiated by production of an RNA primer at a specific site (the origin) on the single-stranded plus-strand template by *E. coli* RNA polymerase in the presence of *E. coli* SSB (8). The origin region contains two hairpin sequences that are involved in the primer synthesis (28, 42). The deletion mutant

TABLE 1. Induction of β-galactosidase in a *dinD*::Mud(Ap *lac*) fusion strain by infection with mutant phages

Phage or agent	β-Galactosidase activity[a] (U/A_{660} unit)
Uninfected	41 ± 10
Wild-type f1	47 ± 14
R377	165 ± 26
M13ΔE101	160*
M13Gori101	67*
Mitomycin C (0.25 μg/ml)	281 ± 15

[a] JH137 cells were grown and either infected with phage as indicated or mixed with mitomycin C as described in the legend to Fig. 4. After incubation for 2 h at 34°C, β-galactosidase ativity was determined as described previously (27). The values represent the averages of data from two (*) or four independent experiments.

R377 lacks the entire region of the minus-strand origin. Conversion of parental single-stranded DNA of R377 to the RF was much slower than that of the wild-type phage (Fig. 3). In accordance with this, during the early stage of infection, accumulation of the R377 single-stranded DNA in the cell was observed, whereas with the wild-type phage no single-stranded DNA was detected (Fig. 7). The estimated amount of intracellular R377 single-stranded DNA at 10 min after infection was approximately two or three molecules per cell.

Late in infection (starting from about 15 to 20 min after infection) with wild-type f1, much larger amounts of single-stranded DNA accumulated in the cell (up to 50 to 100 molecules per cell; Fig. 7). These molecules of single-stranded DNA are bound to gene V protein (gpV) of the phage to form a gpV–single-stranded DNA complex (10, 26), which is a precursor of the progeny phage particle. Since no SOS induction is observed in the wild-type f1-infected cells, the single-stranded DNA complexed with gpV must not induce SOS response. Accumulation of single-stranded DNA and the RF was observed even in R377-infected cells in the late stage of infection, although to lesser extents than in the wild type-infected cells (Fig. 7). Such accumulation of R377 DNA species must require minus-strand synthesis and must be due to a secondary, as-yet-uncharacterized mechanism for minus-strand synthesis. At any rate, it seems that only the single-stranded DNA that accumulates early in infection and is free of gpV (possibly bound to the *E. coli* SSB) can activate RecA protein.

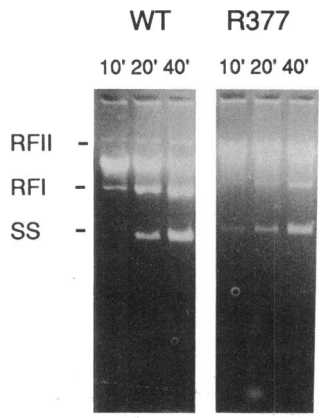

FIG. 7. Intracellular phage DNAs in infected cells. K38 cells were infected with either wild-type f1 (WT) or R377 at a multiplicity of 20. Aliquots (1 ml) of each culture were harvested at various times (as indicated) and lysed, and DNA was extracted and electrophoresed as described in Materials and Methods.

This SOS-inducing system of infection with the mutant filamentous phage might be useful for further studies of the physiology of SOS induction, since the system does not involve any nonspecific effects of UV light or chemical reagents.

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