JOURNAL OF BACTERIOLOGY, Dec. 1993, p. 7887–7900
0021-9193/93/247887-14$02.00/0

Vol. 175, No. 24

A Cytotoxic Early Gene of *Bacillus subtilis* Bacteriophage SPO1

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Received 21 June 1993/Accepted 19 October 1993

Some of the early genes of *Bacillus subtilis* bacteriophage SPO1 were hypothesized to function in the shutoff of host biosyntheses. Two of these genes, *e3* and *e22*, were cloned and sequenced. E22 showed no similarity to any known protein, while E3, a highly acidic protein, showed significant similarity only to other similarly acidic proteins. Each gene was immediately downstream of a very active early promoter. Each was expressed actively during the first few minutes of infection and was then rapidly shut off and its RNA rapidly degraded. An *e3* nonsense mutation severely retarded the degradation of *e3* RNA. Expression of a plasmid-borne *e3* gene, in either *B. subtilis* or *Escherichia coli*, resulted in the inhibition of host DNA, RNA, and protein syntheses and prevented colony formation. However, the *e3* nonsense mutation caused no measurable decrease in either burst size or host shutoff during infection and, in fact, caused an increased burst size at high multiplicities of infection. We suggest that *e3* is one of several genes involved in host shutoff, that its function is dispensable both for host shutoff and for phage multiplication, and that its shutoff function is not entirely specific to host activities.

SPO1 is a large, virulent bacteriophage of *Bacillus subtilis*, in whose genome thymine is completely replaced by hydroxymethyluracil. SPO1 genes have been categorized as early, middle, or late, on the basis of the time at which transcription begins. Early genes are transcribed by the host RNA polymerase, having sigma factor A, and the sequential activation of middle and late genes is mediated by the sequential appearance of the middle-specific sigma factor (specified by SPO1 early gene *28*) and the late-specific sigma factor and accessory (specified by SPO1 middle genes *34* and *33*). To the extent that gene functions are known, DNA replication enzymes are specified by middle genes and structural proteins are specified by late genes (58).

SPO1 specifies at least 21 early proteins, identified as bands on gel electrophoresis and named E1 through E21 in order of decreasing molecular weight (22, 50). The large majority of early transcription takes place within the 12.4-kb terminal redundancy (TR), each copy of which includes at least 13 early promoters, most of which are highly active (3, 48, 51). Except for gene *28*, the functions of the SPO1 early proteins are unknown. We hypothesize that at least some of them are involved in the shutoff of host macromolecular biosynthesis, since shutoff of most host DNA, RNA, and protein syntheses begins so early in infection as to be most easily attributed to the action of early genes (15, 16, 22, 50, 68). The selective advantage obtained from the rapid shutoff of host biosyntheses, which would otherwise compete with the corresponding phage syntheses, offers a plausible explanation for the evolution of this highly active duplicated cluster of early genes. Moreover, the presence of genes whose activity is to shut off essential host functions provides a ready explanation for our earlier observation that most restriction fragments from the TR were unclonable (6).

To understand the mechanisms by which SPO1 shuts off host biosyntheses, we have begun an analysis of the SPO1 early genes. We chose to start with early gene *e3*, because it occupies most of *Eco*RI* fragment 26, which is one of the unclonable fragments from the TR (6, 47). In the course of this analysis, we discovered another gene just upstream of *e3*, overlapping *Eco*RI* fragments 15 and 26, which we named *e22*. Here, we present the nucleotide sequences and expression patterns of genes *e3* and *e22* and show that the *e3* product does have host shutoff activity. However, a strain of SPO1, whose E3 activity has been destroyed by mutation, is not deficient in host shutoff or phage production, suggesting that E3 function is redundant to functions supplied by other SPO1 genes.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* CB-10 and CB-313, suppressor minus and plus, respectively, were described previously (18), as were *Escherichia coli* DH5, 71-18, CJ236, and MV1184 (52).

Plasmids. pDH32 was obtained from D. Henner. It is a modification of the integration vector ptrpBG1 (54) and contains a promoterless *E. coli lacZ* gene in translational fusion to the ribosome binding site of the *B. subtilis* gene *spoVG* (71). pCL2 has a spontaneously derived clonable mutant of SPO1 *Eco*RI* fragment 26 (31), inserted at the *Eco*RI site of pDH32. That fragment has a nonsense mutation at codon 71 of the *e3* gene, which is presumably why it could be cloned. pPW110 is a modification of pDH32, in which the polylinker, just upstream of the *lacZ* gene, has been replaced by a new polylinker, *Eco*RI-*Sst*I-*Kpn*I-*Bgl*II-*Hin*dIII-*Sna*B1-*Bam*HI.

pPW19, our primary expression vector, is shown in Fig. 1. It was formed by introducing a new polylinker into pSI-1, an *E. coli-B. subtilis* shuttle vector provided by D. Henner. It has the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible spac-I promoter, a hybrid of SPO1 promoter $P_{E}5$ and the *E. coli* lactose operator (69), just upstream of the polylinker. Also on this plasmid are the *E. coli lacI* gene, under control of the promoter and ribosome binding site of the *Bacillus licheniformis* penicillinase gene; replication origins from pBR322 (52) and pUB110 (38), permitting replication in either *E. coli* or *B. subtilis*; and the chloramphenicol acetyltransferase gene from pC194 (25), providing chloramphenicol resistance. pPW19 and its derivatives show structural instability (4, 26), so it was

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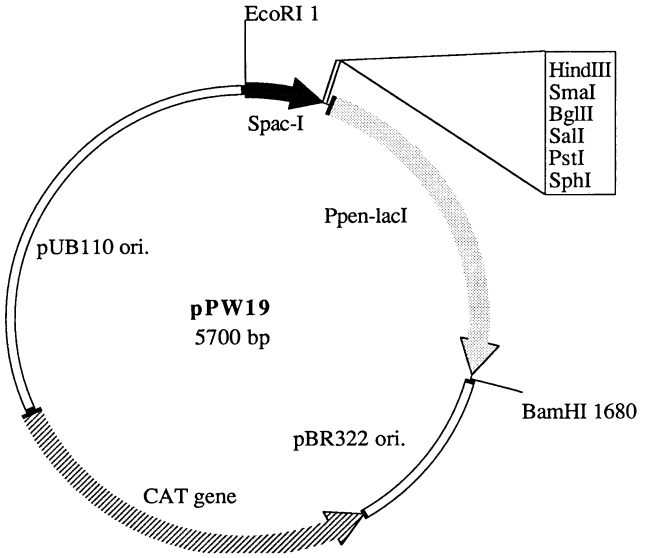

FIG. 1. *E. coli-B. subtilis* expression vector pPW19.

necessary frequently to start cultures from fresh transformants or from aliquots of cultures stored at −70°C and to monitor the integrity of the plasmid.

pPW19-e3, pPW19-e3^{m3}, and pPW19-e3^{m71} have the wild-type *e3* gene or the *e3* gene with nonsense mutations in codon 3 or 71, respectively, inserted at the *Sma*I site of pPW19, oriented such that they are expressed correctly from the spac-I promoter. The *e3* and *e3*^{m3} genes were provided on the 890-bp polymerase chain reaction (PCR) fragment shown in Fig. 2E, made from wild-type or *e3*^{m3} mutant SPO1 DNA, respectively. The cloned fragment carrying the *e3*^{m71} gene was made by PCR from pCL2 by using the same left-end primer used for *e3* and *e3*^{m3} but with the right-end primer based on a vector sequence adjacent to the cloning site. pPW19-lacZ was formed by inserting between the *Hin*dIII and *Sma*I sites of pPW19 a *Hin*dIII-*Sca*I fragment of pPW110 carrying the promoterless *lacZ* gene, oriented such that the *lacZ* gene was correctly expressed from the spac-I promoter. Our laboratory names for the plasmids pPW19-e3, pPW19-e3^{m3}, pPW19-e3^{m71}, and pPW19-lacZ are pPW27, pPW33, pPW28, and pMM3, respectively.

pMS1 is an *E. coli-B. subtilis* shuttle vector, obtained from E. P. Geiduschek (53). pUC19 and pEMBL19(+) were obtained commercially, and pUC118 and pUC119 were obtained from J. Messing. The latter three are phagemids used for generating single-stranded versions of cloned fragments (52). pPW13 has a PCR fragment of the *e3* gene from pCL2 cleaved by *Eco*RI and cloned between the *Sma*I and *Eco*RI sites of pUC118, such that the transcribed strand of the *e3* gene is produced in single-stranded DNA. pPW22wt has the *e3* gene (on the 890-bp fragment shown in Fig. 2E) cloned into the *Sma*I site of pUC19. pAT3-anti has an *Eco*RI fragment of pPW22wt, carrying the *e3* gene, cloned into the *Eco*RI site of pEMBL19(+), such that the transcribed strand of the *e3* gene is produced as single-stranded DNA.

Media and growth conditions. Trypticase soy agar (Becton Dickinson) was used for top and bottom agar at 12 and 40 g/liter, respectively. Tryptose blood agar base (Difco) was used at 15.4 g/liter for top agar from which plaque lifts were made. Liquid-broth media included NY (8 g of nutrient broth per liter, 5 g of yeast extract [Difco] per liter) and VY (59). Defined liquid media included adsorption buffer (0.05 M Tris [pH 7.5], 0.1 M NaCl, 0.01 M $MgSO_4$), Spizizen's salts medium (56), C1 (Spizizen's salts medium plus glucose plus complete amino acids [57]), C4 (C1 lacking leucine, isoleucine, and valine), and C6 (C1 lacking cysteine, methionine, arginine, and lysine). C4/NY is NY medium diluted 1:10 into C4. Ampicillin was used at 50 μg/ml, and chloramphenicol was used at 10 μg/ml for *E. coli* and 5 μg/ml for B. subtilis. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 0.2 mM, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 50 μg/ml.

SPO1 lysates were prepared by infecting *B. subtilis* cells (2×10^8 cells per ml) at a multiplicity of infection (MOI) of 4 to 6 and shaking at 37°C until lysis. Cell debris was removed by centrifugation for 10 min at 7,600 × *g*. The average titer was 1×10^{10} to 2×10^{10} PFU/ml. For concentration and/or removal from broth medium, phage was centrifuged for 150 min at 13,000 × g and resuspended in adsorption buffer.

DNA manipulation and transformation. Routine procedures for preparation and enzymatic manipulation of DNA and for transformation of *B. subtilis* or *E. coli* were performed as described elsewhere (6, 52, 57). Single-stranded DNA was prepared by using the M13KO7 helper phage/MV1184 host cell system (66) as described by Sambrook et al. (52). Double-stranded plasmid DNA for sequencing was prepared as described in the *Promega Protocols and Applications Guide* (48a). DNA bands were recovered from agarose gels with the Prep-A-Gene DNA Purification Matrix Kit (Bio-Rad Laboratories, Richmond, Calif.). Oligonucleotides were obtained from Oligos, Etc., Inc., or were synthesized with a Biosearch 8600 DNA synthesizer and desalted with Econo-Pac 10DG columns (Bio-Rad) according to the manufacturers' instructions.

PCR. PCR was performed with the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, Conn.), following procedures supplied with the kit. For amplification of plasmid DNA, 10 to 100 ng of template was used, the initial denaturation was at 97°C for 7 min, and the program (92°C, 1 min; 54°C, 20 s; 72°C, 30 s) was repeated 30 times. For amplification of hydroxymethyluracil-containing SPO1 DNA, 1 μg of template was used and an additional 5 cycles (92°C, 1 min; 42°C, 20 s; 72°C, 30 s) were performed before the 30 cycles mentioned above. The PCR product was analyzed on a 4% agarose gel (ratio of NuSieve low-melt agarose to Bio-Rad high-strength agarose, 3:1) and recovered from the gel as described above.

DNA sequencing and sequence analysis. Single- and double-stranded DNA sequencing were performed by the dideoxy chain termination method with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Direct sequencing of purified PCR fragments was performed as described by Wang et al. (67). Each sequence was determined at least twice, from both strands. Sequence analysis used the software package provided by the Genetics Computer Group at the University of Wisconsin and the GenBank, EMBL, and SwissProt databases. ΔG values for ribosome binding sites were calculated by using the parameters of Tinoco et al. (64) with procedures exemplified by Rabinowitz and colleagues (39, 42).

Liquid hybridization of pulse-labeled RNAs. *B. subtilis* CB-10 cells, in VY medium at 37°C at approximately 2×10^8 cells per ml, were infected with either wild-type or mutant SPO1 at an MOI of 4 to 6. At various times after infection, aliquots were pulse-labeled for 2 min with 40 μCi of [5,6-^{3}H]uridine (52 Ci/mmol; ICN) per ml and stopped by adding ice-cold NaN_3 (Sigma) to 25 mM. RNA was isolated by the sonication method of Gilman et al. as described by Raleigh et

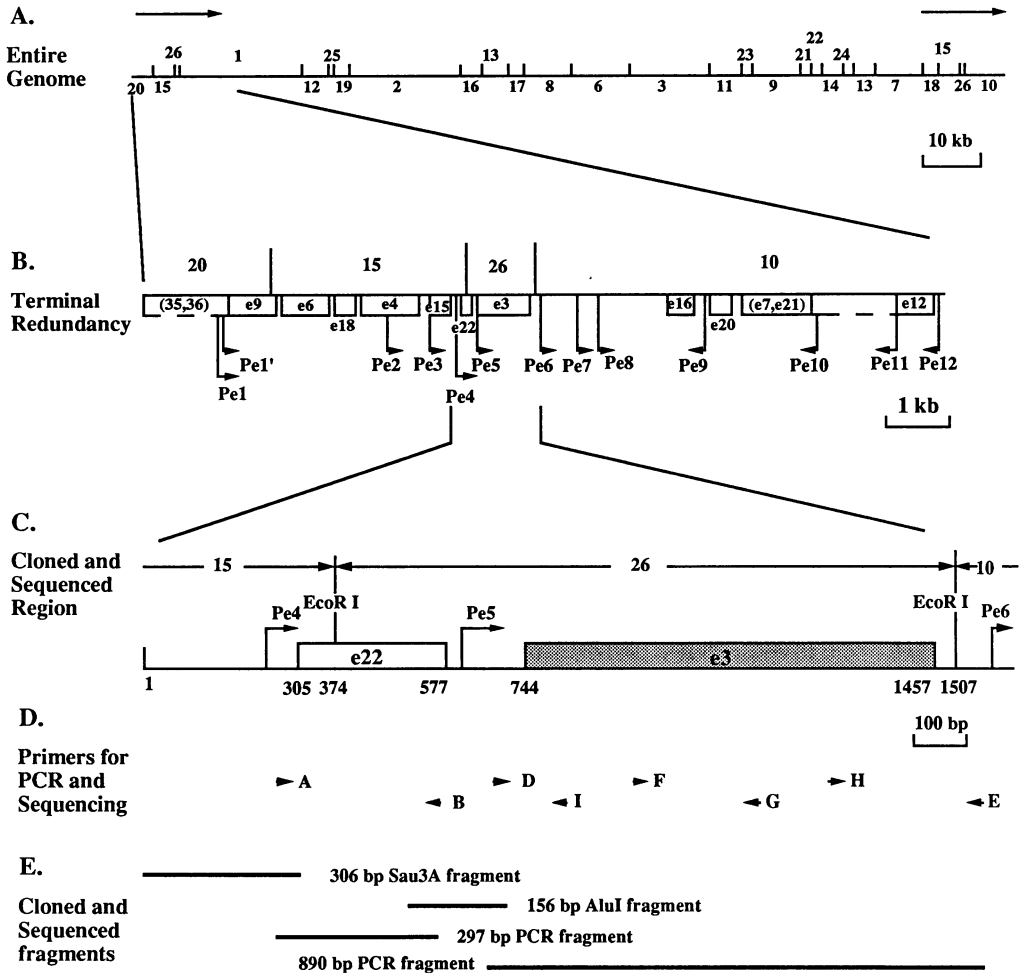

FIG. 2. Maps and sequencing strategy. (A) *Eco*RI* restriction map of the SPO1 genome. Numbers represent *Eco*RI* fragments. Arrows above the map indicate the position of the TR. (B) Map of the TR. *Eco*RI* fragments in the TR are shown by numbers above the map. Arrows and boxes under the map represent the approximate locations of the early promoters (Pe) and the known early genes (e). Arrows show the direction of transcription from each promoter. (C) Map of the region studied in this paper. Arrows and boxes above the line show the positions of early promoters (Pe4 and Pe5) and genes (*e22* and *e3*). Numbers 15, 26, and 10 identify the relevant *Eco*RI* fragments. Numbers below the line indicate the positions, in the sequence shown in Fig. 3, at which the entire sequenced region, the *Eco*RI* fragments, and genes *e22* and *e3* begin and end. (D) Positions of primers used for PCR and sequencing. Arrows indicate the direction of replication from each primer. In the sequence in Fig. 3, the positions of primers A through I, reading 5′ to 3′, are as follows: A, 281 to 298; B, 577 to 559; D, 642 to 659; E, 1531 to 1514; F, 935 to 951; G, 1243 to 1226; H, 1312 to 1328, and I, 826 to 810. (E) Positions of cloned fragments used for sequencing. *Eco*RI* fragments 15 and 26 were cleaved with *Sau*3A and *Alu*I, respectively, and cloned upstream of a promoterless *lacZ* gene in pPW110. Promoter-containing clones were identified as dark blue colonies on X-Gal plates, and the cloned fragments were sequenced. The sequences immediately surrounding promoters Pe4 and Pe5 had been determined previously (20, 32, 33, 69), permitting confirmation that a 306-bp *Sau*3A fragment (nucleotides 1 to 306) contained Pe4 and that a 156-bp *Alu*I fragment (nucleotides 504 to 659) contained promoter Pe5. The sequences presented differ slightly from those previous determinations. The sequence around promoter Pe6, located at the left end of *Eco*RI* fragment 10, was from Greene et al. (20). The regions between the promoters were then amplified by PCR by using primers A and B for nucleotides 281 to 577 and primers D and E for nucleotides 642 to 1531. Several clones were made of the products of each reaction, and several different products of each reaction were sequenced, either by sequencing different cloned fragments (the *e3* gene), or by sequencing one cloned fragment and the direct product of the PCR reaction (the *e22* gene).

al. (49). The newly synthesized *e3* RNA was measured by a modification of the liquid hybridization procedure of Gage and Geiduschek (15). Approximately 3 μg of RNA from each pulse-labeled culture was incubated with an excess amount (5 μg) of single-stranded *e3* DNA (complementary to *e3* RNA, produced from pPW13, a phagemid clone of the *e3* gene) in 400 μl of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 63°C for 16 h. The hybridization mixture was then treated with RNase A (20 μg/ml; from Sigma) at 37°C for 20 min and diluted into 15 ml of 0.5 M KCl in 10 mM Tris-HCl (pH 7.3) before being filtered through a 0.45-μm-pore-size nitrocellulose filter (Millipore). The filter was washed with 30 ml of 0.3% trichloracetic acid (TCA) in 0.1× SSC, dried, and counted with Liquifluor scintillation fluid (NEN Research Products, Boston, Mass.). The background of nonspecific hybridization and binding was measured by treating another aliquot of RNA in the same way except that the *e3*-specific DNA was replaced by an equal quantity of pUC119 DNA. This background was subtracted from the amount of hybridization to *e3*-specific DNA. The value obtained was multiplied by

3.0/(amount [micrograms] of RNA in sample) to normalize for the slight deviation from 3.0 μg in the amount of input RNA in individual samples.

RNA preparation and Northern (RNA) blot analysis. RNA was isolated from *B. subtilis* cells by a modification of the procedure of Curran and Stewart (7). CB-10 cells, grown at 37°C to approximately 2×10^8 cells per ml, were infected with SPO1 at an MOI of 4 to 6. At various times after infection, 1.5-ml aliquots were transferred to microcentrifuge tubes containing cold NaN_3, producing a final NaN_3 concentration of 25 mM. The cells were centrifuged and resuspended in 50 μl of RNA isolation buffer I (15 mM Tris-HCl [pH 8.0], 15% sucrose, 8 mM EDTA) plus 10 mg of lysozyme per ml and incubated at 37°C for 15 min. Diethyl pyrocarbonate (DEPC) (3 μl) and RNA isolation buffer II (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 1 mM sodium citrate, 1.5% sodium dodecyl sulfate [SDS]) (50 μl) were added, and the mixture was incubated at 37°C for 5 more minutes. It was then chilled on ice for 2 min, and 25 μl of H_2O saturated with NaCl were added. The mixture was kept at −20°C for 10 min before centrifuging at 12,700 × *g* for 10 min. The supernatant was extracted once with an equal volume of sevag mix (ratio of chloroform to isoamyl alcohol, 24:1) and precipitated with 3 volumes of 100% ethanol. The RNA pellet was washed twice with 80% ethanol, dried, and dissolved in an appropriate volume of DEPC-treated water.

Northern blots and hybridization were performed as described by Sambrook et al. (52). Portions (5 μg) of each RNA sample were subjected to electrophoresis on 2.2 M formaldehyde-1.1% agarose gels (2 V/cm for 4 h). The gel was then stained with 0.5 μg of ethidium bromide per ml for 30 min and destained in water for 30 min before photography. The RNA was transferred to a Duralon-UV membrane and cross-linked to the filter by using a Stratalinker UV Crosslinker according to the manufacturer's instructions (Stratagene, La Jolla, Calif.). Hybridization probes used the 890- and 297-bp PCR fragments (Fig. 2E) for *e3* and *e22*, respectively. They were labeled with [^{32}P]ATP (5,000 μCi/mM; NEN) by using a Random Primer DNA Labelling Kit (Boehringer Mannheim) according to the manufacturer's instructions. The specific activities of the probes were about 10^9 cpm/μg. The filters were prehybridized for 4 h at 60°C in 10 ml (10× Denhardt's solution, 50 mM Na_3PO_4, 0.5% SDS, 0.2× SSC). Denatured probe (0.1 μg) was added, and the hybridization proceeded overnight at 60°C. The filters were washed twice in 2× SSC for 10 min at room temperature and twice in 0.2× SSC-0.1% SDS for 20 min at 60°C. Autoradiography was done with XAR-5 X-ray film. Densitometry was performed with a Molecular Dynamics computing densitometer.

Protein labeling and electrophoresis. CB-10 cells, grown in C6 medium at 37°C to approximately 2×10^8 cells per ml, were infected at an MOI about 5 with SPO1, which had been resuspended in adsorption buffer. At various times before and during infection, 1-ml aliquots were pulse-labeled for 2 min with 25 μCi of [^{35}S]Met-Cys (1,140 Ci/mmol; Amersham). Alternatively, CB-10 cells carrying plasmid pPW19-e3 were pulse-labeled after various times of IPTG induction. Cells were lysed and proteins were extracted as described by Heintz and Shub (22). Portions (15 μl) of each protein extract were loaded on an SDS–12% polyacrylamide gel and separated by electrophoresis (SDS-PAGE) as described by Sambrook et al. (52). The gel was dried and autoradiographed for 5 h at room temperature. Protein molecular weight markers were purchased from Bio-Rad.

Mutagenesis of the *e3* gene. Site-directed mutagenesis by the procedure of Kunkel et al. (28) used the Muta-Gene Phagemid In Vitro Mutagenesis Kit (Bio-Rad) according to the manufacturer's instructions. Phagemid pAT3-anti [a clone of the *e3* gene in phagemid pEMBL19(+)] was used to prepare the uracil-containing single-stranded DNA template. The 33-mer mutagenic oligonucleotide 5′-AGATTTCAAATGGCTTAGT CAAATAACGTGTAC-3′ represents nucleotides 735 to 767 of the sequence shown in Fig. 3. The two mismatches are underlined. The mutation converted codon 3 of the *e3* gene, AAA, into the nonsense codon TAG, as confirmed by DNA sequencing. We have named this mutation $e3^{m3}$.

Introduction of the $e3^{m3}$ mutation into SPO1. The procedure followed was a modification of that employed by Sayre and Geiduschek (53). An *Eco*RI fragment of the mutant pAT3-anti, carrying the *e3* gene with the $e3^{m3}$ mutation, was cloned into the *Eco*RI site of pMS1, an *E. coli-B. subtilis* shuttle vector. This recombinant plasmid was transformed into CB-313, a *B. subtilis* suppressor strain, which inserts lysine at UAA or UAG codons (41). These transformants, growing in VY plus 5 μg of chloramphenicol per ml, were infected by wild-type SPO1 at an MOI of about 1.0. After 15 min, the cultures were diluted 50-fold into VY plus chloramphenicol. At 45 min later, 0.02 ml of $CHCl_3$ per ml of culture was added, shaking was continued for 1 more minute, and progeny phage was plated on a CB-313 lawn with 15.4 g of tryptose blood agar base per liter as top agar. Recombinants that had incorporated the $e3^{m3}$ mutation were identified by filter hybridization. Duplicate plaque-lift filters were made (by using BA85 filters; Schleicher and Schuell) and probed by mutant and wild-type oligonucleotides (5′-AAATGGCTTAGTCAAATAA-3′ and 5′-AAATGGCTAAATCAAATAA-3′, respectively). The oligonucleotides were end labeled with [γ-^{32}P]ATP (4,500 Ci/mmol; Amersham) and purified as described by Sambrook et al. (52), yielding a specific activity of 7×10^9 to 10×10^9 cpm/μg. Five filters, each with approximately 1,200 lifted plaques, were incubated with 20 ml of prehybridization buffer at 42°C for 4 h and then with 10 ml of hybridization buffer plus 2×10^6 cpm of the appropriate oligonucleotide probe per ml at 42°C for 16 h. Filters were washed twice with 100 ml of 6× SSC at room temperature for 5 min (low stringency) and then washed individually in 300 ml of 6× SSC in a 400-ml beaker at 38°C for 2 min (high stringency). Of about 12,000 plaques tested, 98 hybridized to the mutant probe, implying a replacement frequency of about 0.4% (considering that each genome has two target sites for integration). The lengths of the regions of homology between the plasmid and the SPO1 genome were 108 and 760 bp to the left and right of the mutation site, respectively. Each of the 98 plaques also hybridized to the wild-type probe, presumably because the mutation had replaced the wild-type allele in only one of the redundant regions. To permit segregation of homozygous mutants (expected in the normal course of replication and resolution of concatemers during SPO1 infection [5]), some of these plaques were propagated through another round of infection of CB-313, and progeny in which the $e3^{m3}$ mutation had replaced the wild-type allele in both copies of the TR were identified by the same filter hybridization procedures.

DNA, RNA, and protein synthesis assays. Since efficient incorporation of ^{3}H-labeled leucine could not be accomplished in media with high concentrations of leucine, all experiments involving pulse-labeling of DNA, RNA, or protein with tritiated precursors in SPO1-infected cells were done in C4 medium by using infecting phage that had been resuspended in adsorption buffer. The additional stress caused by the plasmid-borne *e3* gene, even without induction, caused DH5(pPW19-e3) to grow very slowly in C4 medium. Therefore, all such experiments on plasmid-containing strains were done in C4/

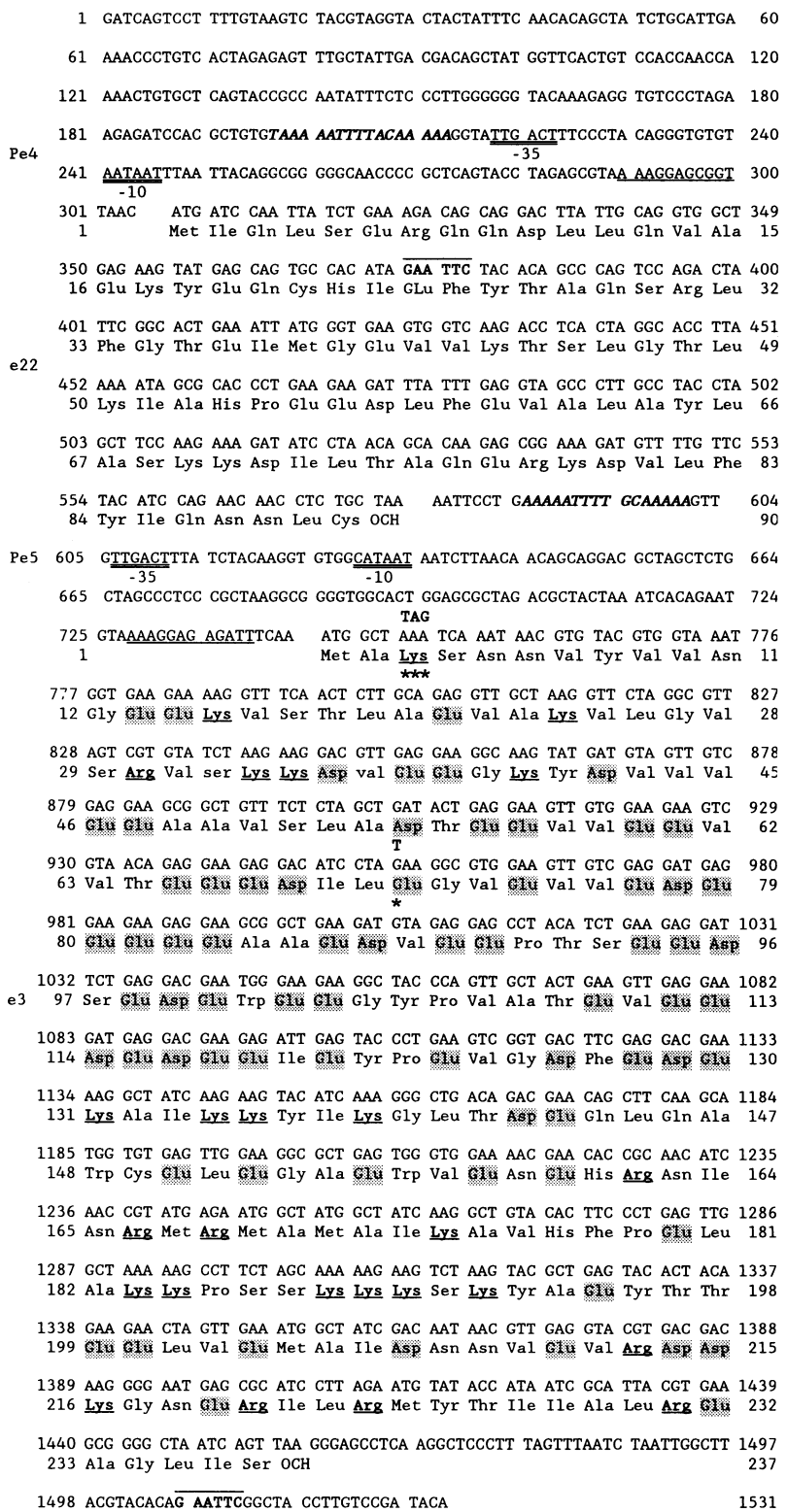

FIG. 3. Complete sequence of the 1.5-kb cloned segment. The −10 and −35 regions of promoters Pe4 and Pe5 are indicated by double underlines, ribosome binding sites are indicated by single underlines, and *Eco*RI sites are indicated by bold letters, overlined. The regions just upstream of the −35 sites, having runs of adenines with 11-nucleotide periodicity, are shown in bold italics. The transcription initiation sites (and thus the locations of the −10 and −35 sites) were previously identified by RNA sequencing (32, 33). The translation initiation sites have not been independently confirmed by amino acid sequencing. The deduced amino acid sequences of E3 and E22 are shown under their nucleotide sequences. In E3, the strongly acidic and basic amino acids are shown by stippling and boldface underlines, respectively. Nucleotide substitutions in the mutations discussed in the text are shown above the wild-type sequences that they replace and are marked by an asterisk under the wild-type sequences.

NY. Pulse-labeling was for 3 min with 0.8 μCi of the appropriate radioactive precursor per ml in 0.5-ml aliquots of the culture being tested, and the time shown for each was the beginning of the 3-min period. Host DNA was labeled with [5-^{3}H]thymidine (49 Ci/mmol; ICN), host and/or phage RNA was labeled with [5-^{3}H]uridine (29 Ci/mmol; Amersham), and host and/or phage protein was labeled with [4,5-^{3}H]leucine (80 Ci/mmol; ICN). Each pulse was terminated by precipitation with 5% TCA. Precipitates were collected on 0.45-μm-pore-size nitrocellulose filters, which were washed, dried, and counted as described above. For leucine incorporation, the TCA precipitates were boiled for 15 min and then kept on ice for at least 10 min before filtration. Phage DNA was labeled with [5,6-^{3}H]uridine (52 Ci/mmol; ICN). Each pulse was terminated by adding 0.5 ml of cold 2.0 N KOH. The samples were incubated for 6 h at 37°C, neutralized with 0.5 ml of 2.0 N HCl, and precipitated with TCA as described above.

Nucleotide sequence accession number. The DNA sequence reported here has been submitted to GenBank and assigned accession number L20825.

RESULTS

Structure of SPO1 genes *e22* and *e3*. Since intact *Eco*RI* fragments 15 and 26 were unclonable, subfragments, with genes separated from promoters, were cloned and sequenced as described for Fig. 2. The combined fragments constituted a continuous segment of 1.5 kb, containing all of *Eco*RI* fragment 26 and the right end of *Eco*RI* fragment 15 (Fig. 2C). The sequence of this 1.5-kb segment is shown in Fig. 3. Its two early promoters, Pe4 and Pe5, were immediately upstream of genes *e22* and *e3*, which specified proteins of 90 and 237 amino acids, respectively. Each open reading frame was preceded by a strong ribosome binding site (40), and each promoter included periodic runs of adenines just upstream of the −35 site.

Of the amino acids in the E3 protein, 30% were acidic, including 55 glutamates and 16 aspartates. These were concentrated in the region from amino acids 46 through 130, which included 46 acidic and no basic amino acids. This acidic region of the E3 protein showed significant similarity to segments of a variety of other proteins, all based primarily on similar concentrations of glutamate residues. No sequences presently in the standard data bases show significant similarity to any other portions of E3 or to any portion of E22.

Regulation of genes *e22* and *e3*. Synthesis of E3 protein began very early in infection, reached a brief peak, and was then shut off more gradually (Fig. 4). The E3 protein was one of the earliest and more abundant SPO1 proteins. Northern blots show that transcription of both *e22* and *e3* was turned on early and turned off a few minutes later and that both RNAs were degraded rapidly (Fig. 5).

Infection with $e3^{m3}$, an *e3* nonsense mutant, resulted in the persistence of *e3* RNA until much later in infection. (The much smaller effect in the same direction on *e22* RNA may or may not be significant.) The persistence of *e3* RNA in $e3^{m3}$ infection could be explained either by autogenous regulation of *e3* transcription or by a direct or indirect effect of the nonsense mutation on the stability of *e3* RNA. To distinguish between these possibilities, *e3* RNA was pulse-labeled at various times after infection (Fig. 6). By 10 min after infection, *e3* RNA synthesis had been shut off as effectively in the mutant infection as in the wild type, showing that the persistence of *e3* RNA in the mutant was not due to continued transcription and thus must be due to increased stability. Such increased stability could also explain the shape of the earlier part of the curve,

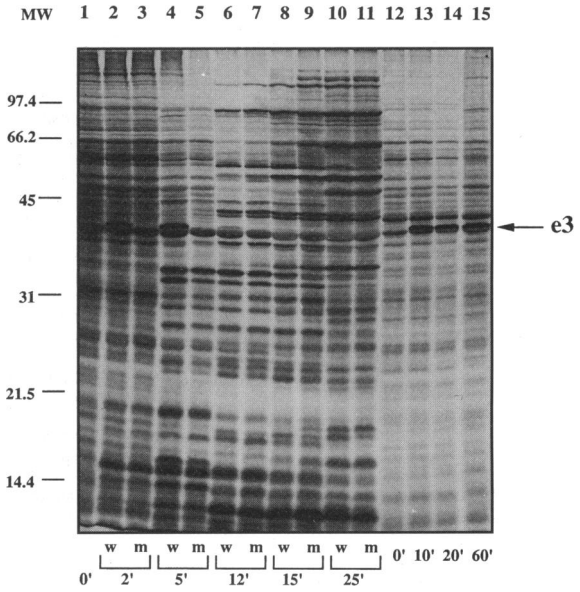

FIG. 4. Electrophoretic analysis of E3 protein synthesis. Cells of CB-10 or CB-10(pPW19-e3), with or without SPO1 infection and with or without induction by IPTG were pulse-labeled with ^{35}S-amino acids for 2 min, starting at the time indicated for each lane, and were analyzed following the procedures described in Materials and Methods. Lanes 1 through 11 show the effect of infection with SPO1 wild-type or with the *e3* nonsense mutant $e3^{m3}$: lane 1, uninfected CB-10 cells; lanes 2, 4, 6, 8, and 10, CB-10 cells infected with wild-type SPO1 (w) for 2, 5, 12, 15, and 25 min, respectively, before pulse-labeling; lanes 3, 5, 7, 9, and 11, CB-10 cells infected with *e3* nonsense mutant SPO1 $e3^{m3}$ (m) for 2, 5, 12, 15, and 25 min, respectively, before pulse-labeling. Lanes 12 through 15 show the effect of induction of the plasmid-borne *e3* gene: lane 12, CB-10(pPW19-e3) cells, uninduced; lanes 13, 14 and 15, CB-10(pPW19-e3) cells induced with 0.2 mM IPTG for 10, 20, and 60 min, respectively, before pulse-labeling. The E3 band (shown by the arrow) was identified both by the position of the induced band in lanes 13 to 15 and by the band, at the same position, that is absent in infection by the *e3* nonsense mutant. Molecular weight (in thousands) is indicated at the left.

where *e3* RNA synthesis was actively proceeding. If the half-life of *e3* RNA in wild-type infection was sufficiently short relative to the length of the pulse, net incorporation in wild-type infection would be reduced relative to that in the mutant, where the half-life was longer. This was, in fact, the result seen at all but the earliest time point. (The distinction between the earliest time point and those immediately following was observed consistently. A possible explanation will be discussed below.)

Cytotoxicity of *e3* gene product. The *e3* gene could be cloned under repressed conditions in the *E. coli-B. subtilis* expression vector pPW19 (Fig. 1). Expression of the cloned *e3* gene by induction with IPTG prevented colony formation in either *E. coli* or *B. subtilis* (Table 1). No such effect was observed when the *e3* gene was replaced by either the *lacZ* gene or an *e3* gene with a nonsense mutation in codon 3. The *e3* gene from a spontaneous clonable mutant of *Eco*RI* fragment 26 (31) caused a small reduction in colony-forming efficiency when expressed from the same promoter. Nucleotide sequencing showed that this mutant had a nonsense mutation at codon 71 of the *e3* gene, suggesting that the 70-amino-acid N-terminal peptide retained some killing activity. Because of greater instability of the plasmid in *B. subtilis*, the wild-type *e3* gene had a greater effect on *E. coli* than on *B. subtilis*.

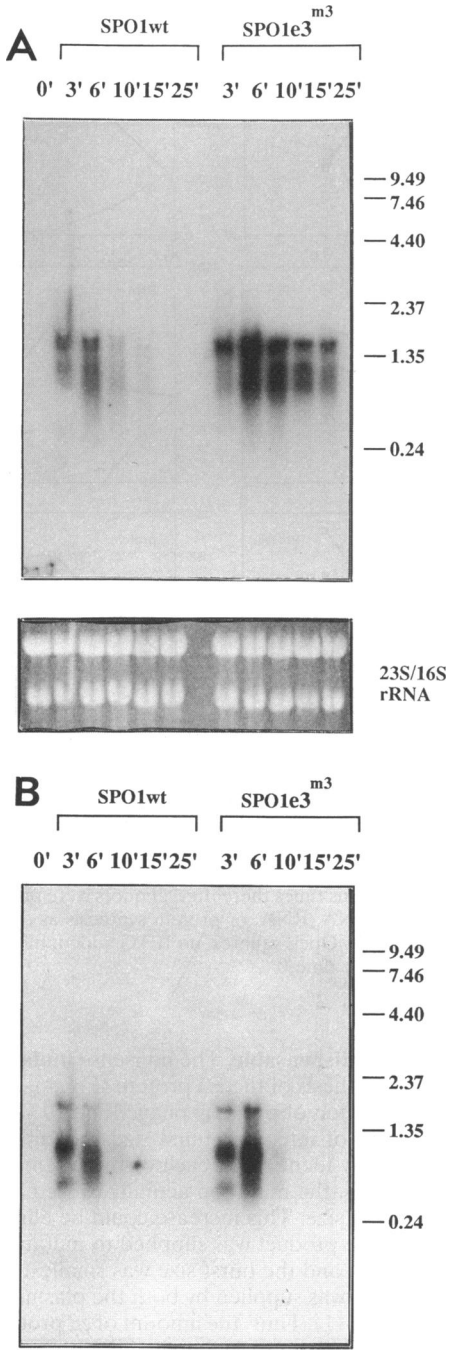

FIG. 5. Northern blots of *e3* and *e22* RNA. CB-10 cells were infected with wild-type SPO1 or the *e3* nonsense mutant SPO1 $e3^{m3}$ and shaken at 37°C until the time indicated by the number at the top of each lane. RNA was extracted and subjected to electrophoresis on a formaldehyde–1.1% agarose gel, as described in Materials and Methods. Molecular size (in kilobases) is indicated at the right of each panel. (A, lower panel) The gel was photographed after ethidium bromide staining and before transfer to show that the amounts of RNA in all lanes were approximately equal. (A, upper panel) The gel was transferred to a nylon filter and probed with the ^{32}P-labeled 890-bp PCR fragment containing the *e3* structural gene (Fig. 2E). (B) The same filter was washed and rehybridized with the ^{32}P-labeled 297-bp PCR fragment containing the *e22* structural gene (Fig. 2E).

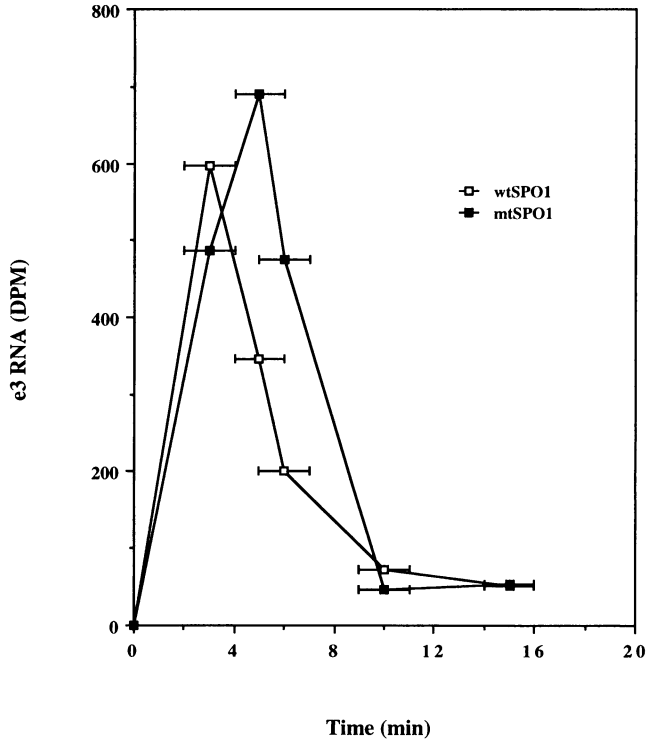

FIG. 6. Pulse labeling of *e3* RNA. CB-10 cells infected with wild-type SPO1 (open squares) or mutant $e3^{m3}$ (closed squares) were pulse-labeled with [^{3}H]uridine for 2 min (represented by the horizontal bars) at various times during infection. The amount of newly synthesized *e3* RNA was determined by hybridizing to *e3*-specific single-stranded DNA and measuring RNase-resistant disintegrations per minute, as described in Materials and Methods.

Figures 7 and 8 show the effects on *E. coli* DH5 when expression of *e3* was induced during exponential growth. Cell growth virtually ceased, and incorporation of radioactive precursors into DNA, RNA, and protein was gradually shut off. Figure 9 shows a similar effect on *B. subtilis* CB-10, although

TABLE 1. Effect of *e3* expression on colony formation

Plasmid present[a]	Ratio for indicated bacterial strains[b]	
	E. coli DH5	*B. subtilis* CB-10
pPW19	0.93	0.97
pPW19-e3	9.6×10^{-6}	2.9×10^{-3}
pPW19-$e3^{m3}$	1.09[c]	0.85
pPW19-$e3^{m71}$	0.12	0.47
pPW19-lacZ	0.98	1.24

[a] Each plasmid name includes the name of the gene, if any, that has been cloned into pPW19 so it is expressed from the IPTG-inducible spac-I promoter. e3 is the wild-type *e3* gene; $e3^{m3}$ and $e3^{m71}$ are mutant *e3* genes, with nonsense mutations at codons 3 and 71, respectively; lacZ is the *E. coli lacZ* gene in translational fusion with a *B. subtilis* ribosome binding site.

[b] Each plasmid was introduced into both *E. coli* DH5 and *B. subtilis* CB-10, and the strains produced were plated with or without IPTG. The values shown are the ratios of the number of colonies formed in the presence of IPTG to the number of colonies formed without IPTG.

[c] DH5 contains the suppressor SupE, which inserts glutamine at UAG codons. The inactivation of *e3* function by the $e3^{m3}$ mutation, even in this strain, suggests that the substitution of glutamine for lysine at position 3 inactivates *e3* function and/or that the suppressed level of E3 protein is insufficient to cause host killing.

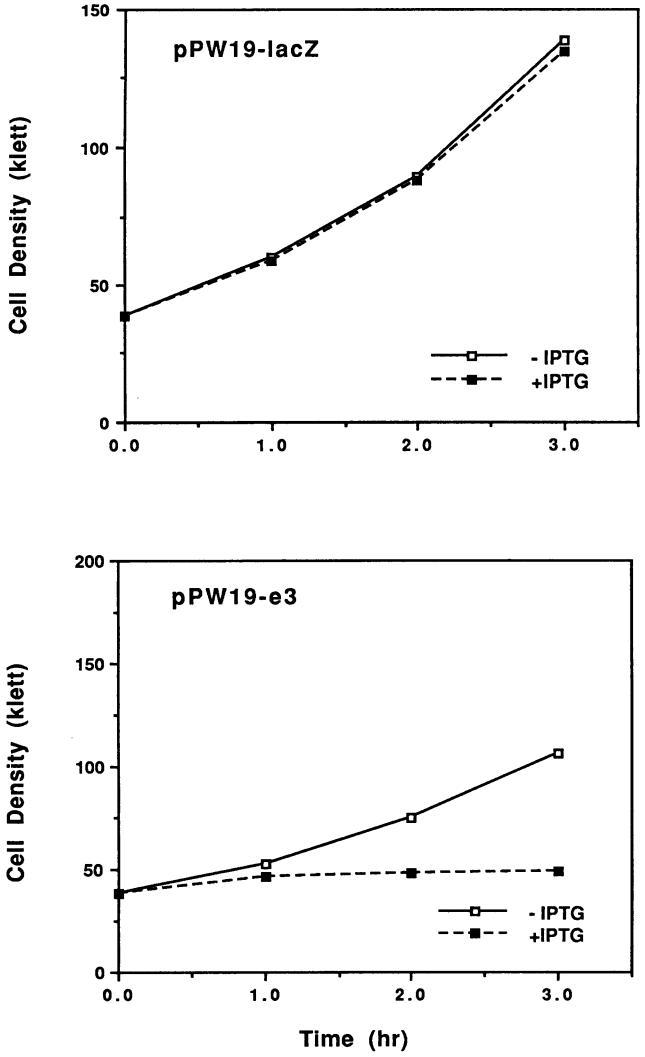

FIG. 7. Effect of induction of E3 on growth of *E. coli*. DH5 cells carrying pPW19-e3 or pPW19-lacZ were grown at 37°C to early exponential phase (about Klett 30). The cultures were split, and 0.2 mM IPTG was added to one-half of each culture at time 0. Continuing growth was monitored by Klett turbidimetry.

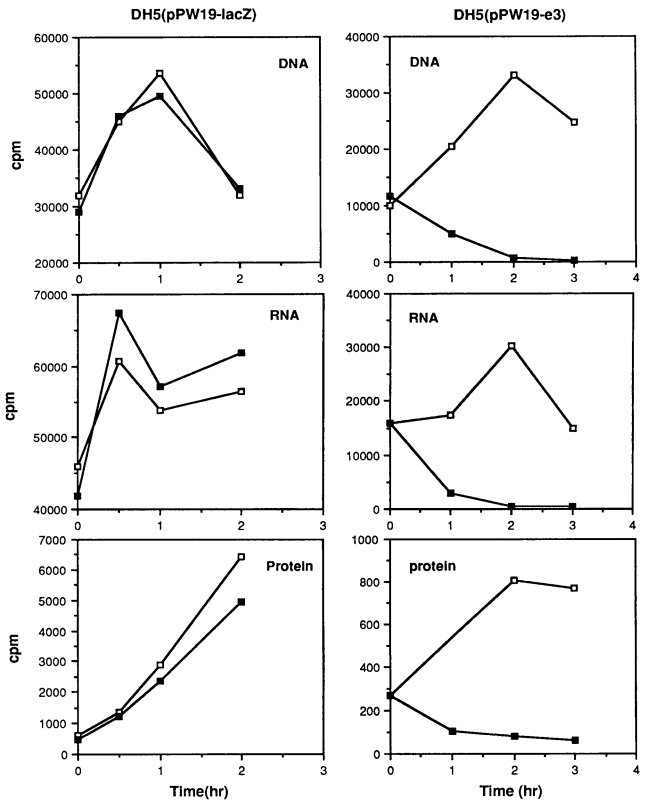

FIG. 8. Effect of *e3* expression on DNA, RNA and protein syntheses in *E. coli*. Exponentially growing cultures of DH5(pPW19-lacZ) (left column) and DH5(pPW19-e3) (right column) were each split into two cultures. At time 0, 0.2 mM IPTG was added to one of each pair of cultures, and at various times thereafter, aliquots were pulse-labeled for measurement of DNA, RNA, or protein synthesis as described in Materials and Methods. Open squares, no IPTG added; filled squares, 0.2 mM IPTG added at time 0.

the shutoffs were not as complete. Again, no such effects were seen when *lacZ* was induced instead of *e3*. [Because the uninduced spac-I promoter was less completely repressed in *E. coli* DH5 than in *B. subtilis* (data not shown), resulting in slower growth for DH5(pPW19-e3) than for DH5(pPW19-lacZ) even without IPTG, and because the pPW19-based plasmids were structurally less stable in *B. subtilis* than in *E. coli*, we do not wish to attribute any significance to the quantitative differences between *E. coli* and *B. subtilis* seen in these figures.]

Although the rate at which the E3 protein was produced in response to IPTG induction of the cloned *e3* gene was roughly comparable to that seen in infection of CB-10 with wild-type SPO1 (Fig. 4), the shutoffs seen in Fig. 8 and 9 occurred much more slowly than those that occur during infection (the latter can be seen in Fig. 12). Thus, if shutoff of host macromolecular synthesis is the natural role of the *e3* gene product during SPO1 infection, the *e3* product is probably not the only component of the shutoff machinery.

The E3 protein is dispensable. The nonsense mutation $e3^{m3}$, which prevented synthesis of the E3 protein (Fig. 4), caused no deficiency in production of progeny phage. Fig. 10 shows that, at low multiplicities of infection, burst sizes of wild type and mutant were virtually identical at each of three temperatures. At high multiplicities, the mutation actually caused a substantial increase in burst size. This increase could be eliminated if the wild-type *e3* gene product was supplied to mutant-infected cells from a plasmid, and the burst size was smallest when the wild-type *e3* product was supplied by both the plasmid and the infecting phage (Fig. 11). Thus, the amount of *e3* product made at a high MOI is apparently enough to be inhibitory, directly or indirectly, to the phage as well as to the host.

The *e3* nonsense mutation also had no consistently observable effect on shutoff of host macromolecular syntheses. Figure 12 shows that DNA, RNA, and protein syntheses were shut off to approximately the same extent by the mutant as by wild-type SPO1. Host DNA synthesis, which was measured independently of phage DNA synthesis by measuring [^{3}H]thymidine incorporation, was shut off virtually completely by either wild-type or mutant infection. (The normal rise in phage DNA synthesis was also unaffected by the mutation.) The RNA and protein synthesis assays did not distinguish host RNA and protein syntheses from the comparable phage syntheses, and the measured decrease in total RNA or protein synthesis was

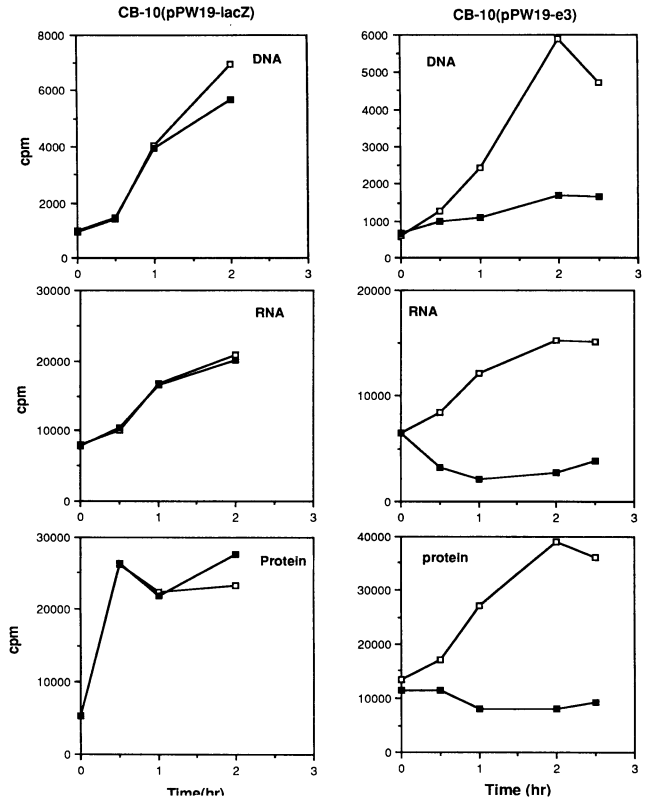

FIG. 9. Effect of *e3* expression on DNA, RNA, and protein syntheses in *B. subtilis*. Exponentially growing cultures of CB-10(pPW19-lacZ) (left column) and CB-10(pPW19-e3) (right column) were each split into two cultures. At time 0, 0.2 mM IPTG was added to one of each pair of cultures, and at various times thereafter, aliquots were pulse-labeled for measurement of DNA, RNA, or protein synthesis as described in Materials and Methods. Open squares, no IPTG added; filled squares, 0.2 mM IPTG added at time 0.

only about 50%. Thus, it remains possible that the $e3^{m3}$ mutation affected the shutoff of some subset of host RNA and/or protein syntheses and that that effect was obscured by the concomitant increase in phage-specific synthesis. However, Fig. 4 shows no indication of any host protein band that was shut off less completely in mutant infection than in wild-type infection, so there was no evidence for any such subset.

DISCUSSION

Structures of genes *e3* and *e22*. The nucleotide sequence of a 1.5-kb segment of the SPO1 terminal redundancy, including the early promoters P_E4 and P_E5 and the early genes *e22* and *e3*, was determined. The sequences of P_E4 and P_E5 had been determined previously (20, 32, 33, 69), and each was known to be very strong (31, 33, 51, 63). Each has, just upstream of its −35 region, two strings of 5 adenines 6 bp apart, the sort of structure that has been shown to be essential for maximal activity of certain promoters, possibly because of the curvature that it imparts to the DNA (2, 12, 36, 37). Indeed, deletion of this region from P_E5 reduced its activity by about 80% (our unpublished results). The Alu 156 promoter, one of the primary promoters used for the study of such structures (36, 37), is from the closely related phage SP82 and is so nearly identical to P_E5 that it is likely that the two promoters were descended from the same promoter in a common ancestor. The ribosome binding sites of *e22* and *e3* have sequences typical of those known to provide efficient translation in *B. subtilis*, on the basis both of the calculated ΔG value (−18.0 and −19.0 for *e22* and *e3*, respectively) and of similarity of sequence and spacing (40).

Gene *e22* specifies a 90-amino-acid protein which shows no significant similarity to any known protein. (We have no direct evidence that *e22* actually is translated, but we assume that it is because of its precise placement just downstream of an efficient promoter and ribosome binding site and because P_E5 is placed as close to it as possible without overlapping it.) The 237-amino-acid E3 protein includes a strongly acidic central region (46 acidic and no basic amino acids in a stretch of 85 amino acids). The only significant similarities between E3 and other known proteins are between this acidic region and glutamate-rich regions of a variety of other proteins. The roles of such regions in most such proteins are not well understood. Activities displayed by or inferred for significant numbers of these proteins include chromosome binding (in at least some cases for regulation of transcription) (11), Ca^{2+}-binding (30), and/or interaction with other proteins. Those proteins showing the most significant similarities to the acidic region of E3 (E value less than 0.001 when analyzed by the BLAST program [1, 21]) included mouse nucleolar transcription factor 1 (23), varicella-zoster virus alpha *trans*-inducing factor (8), human or mouse major centromere autoantigen B (61), pseudorabies virus gene RSp40 (70), *Drosophila* troponin T (14), *Plasmodium* glutamic-acid-rich protein (65), human sarcoplasmic reticulum histidine-rich calcium-binding protein (24), rabbit ryanodine receptor (62), and bovine prothymosin α (46). Such diversity precludes drawing any conclusions about E3 function on the basis of its similarity to other proteins.

Regulation of the activity of genes *e22* and *e3*. Each of the genes *e22* and *e3* was turned on early in infection and was at peak activity for only a few minutes before shutoff occurred. The E3 protein was one of the few SPO1 proteins detectable by the earliest pulse and for a brief period was one of the more actively synthesized. From the Northern blots, it appears that *e22* was transcribed at least as early as *e3*. The rapid shutoff of *e22* may have been complete, but small quantities of *e3* RNA and protein synthesis remained detectable as late as 15 min after infection.

Infection with the $e3^{m3}$ nonsense mutant resulted in increased levels of *e3* RNA that persisted until late in infection. Pulse-labeling experiments showed that this was not due to continued synthesis at late times in $e3^{m3}$ infection and therefore that the mutation must cause increased stability of *e3* RNA. At several early time points, pulse-labeling did show greater net incorporation in mutant than in wild-type infection. This can be explained by the difference in stability, as discussed above and below, but the possibility that part or all of that greater incorporation could be due, instead, to increased synthesis of *e3* RNA is not excluded. However, any such increase would be much too small to account for the greater accumulation and persistence of *e3* RNA in the mutant. The maximum difference between mutant and wild type, in net incorporation during a 2-min pulse, was 2.4-fold; the average difference, for pulses showing substantial incorporation, was 1.7-fold. Thus, if there were no difference in stability, and even if the maximum differences in synthesis were sustained throughout infection, the difference in synthesis could account for, at most, a 2.4-fold difference in total accumulation. By densitometry of the Northern blot for Fig. 5, the differences in total accumulation of *e3* RNA at 3, 6, 10, 15, and 25 min were estimated to be 1.1-, 6.4-, 46-, 52-, and 169-fold, respectively. Clearly, most of the effect must be due to increased stability

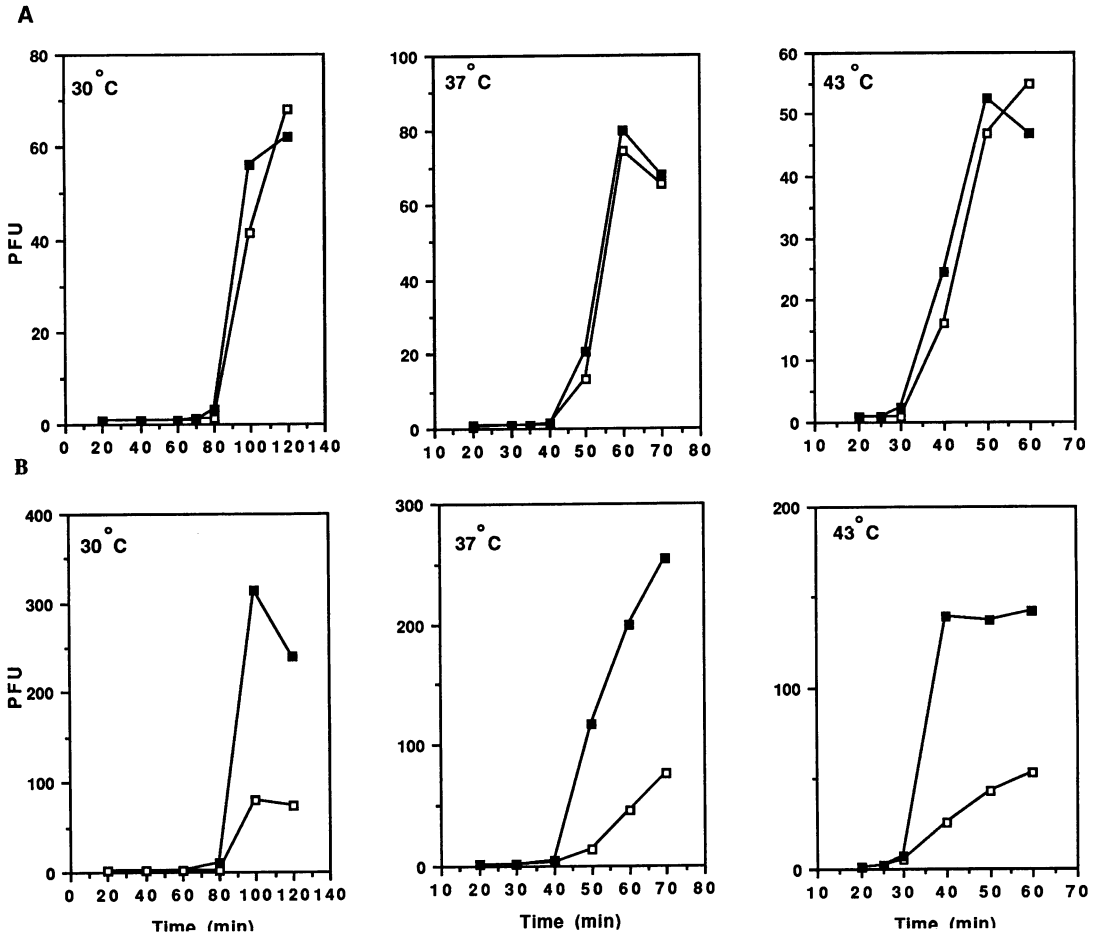

FIG. 10. Effect of the $e3^{m3}$ mutation on progeny phage production. Exponential-phase cultures of CB-10, a nonsuppressing strain of *B. subtilis*, were infected with wild-type SPO1 (open squares) or mutant $e3^{m3}$ (closed squares) at multiplicities of 0.01 (A) or 5 (B). After 10 min, the cultures were diluted 1:2 into VY containing anti-SPO1 antibody. After being shaken for 5 more min, the cultures were diluted $1:10^5$ (for a low MOI) or $1:10^7$ (for a high MOI) into fresh VY, the shaking was continued at the same temperature, and the cultures were assayed periodically for plaque-forming units, which are shown as a function of time after infection.

caused by the $e3^{m3}$ mutation. Since all of the effect can be explained by increased stability, parsimony would suggest that the mutation has no effect on synthesis.

The increased stability could be either because the *e3* product is required, directly or indirectly, for the degradation of *e3* RNA or because the premature termination of translation (or the presence of the two mutant nucleotides) makes the mutant *e3* RNA intrinsically more stable. There are two arguments, neither decisive, in favor of the former explanation. First, nonsense mutations, particularly near the beginning of a cistron, have been known to cause decreased rather than increased stability of the RNA in which they are located (43). Second, pulses which began 4, 5, or 6 min after infection showed greater labeling in the mutant than in the wild-type infection, which could be explained if the net incorporation of label were limited by degradation that occurred in the wild-type but not in the mutant infection. If that is the correct explanation, the degradation must have been more active at those times than during pulses that started at 2 min after infection. That is exactly what would be expected if the *e3* product were required for degradation, since there would be much more *e3* product present at the later times. Of course, there are alternative explanations that have not been excluded, so these arguments are far from conclusive.

Careful examination of the Northern blots in Fig. 5 suggests that, for the most part, the RNAs that hybridized to the *e22* probe did not hybridize to the *e3* probe. Thus, transcripts that began at P_E4 or at any promoter upstream of P_E4 apparently either were terminated between *e22* and *e3* or were cleaved so as to separate the *e22* and *e3* sequences. In vitro transcription studies did not identify a termination site in this region (3), and although there are several sites there capable of forming stem-loop structures, none has the adjacent string of uracils characteristic of efficient rho-independent transcription terminators. It is known that SPO1 early transcripts are processed by the activity of a host enzyme, which cleaves at specific sites to form the smaller RNA molecules found in extracts of infected cells (9, 45). It is possible that one of the stem-loop structures in the region between *e22* and *e3* provides a site for such cleavage, although each of them differs substantially in length and sequence from the stem-loop structures of the three cleavage sites whose sequence is known.

Possible role of early genes in host shutoff. The SPO1 terminal redundancy includes at least 12, and possibly as many as 20, active early genes of unknown function. We suspect that many of these are involved in host shutoff, for the following reasons. (i) Host shutoff begins too early to be accounted for by middle gene activity (15, 16, 22, 50). (ii) The shutoff of host

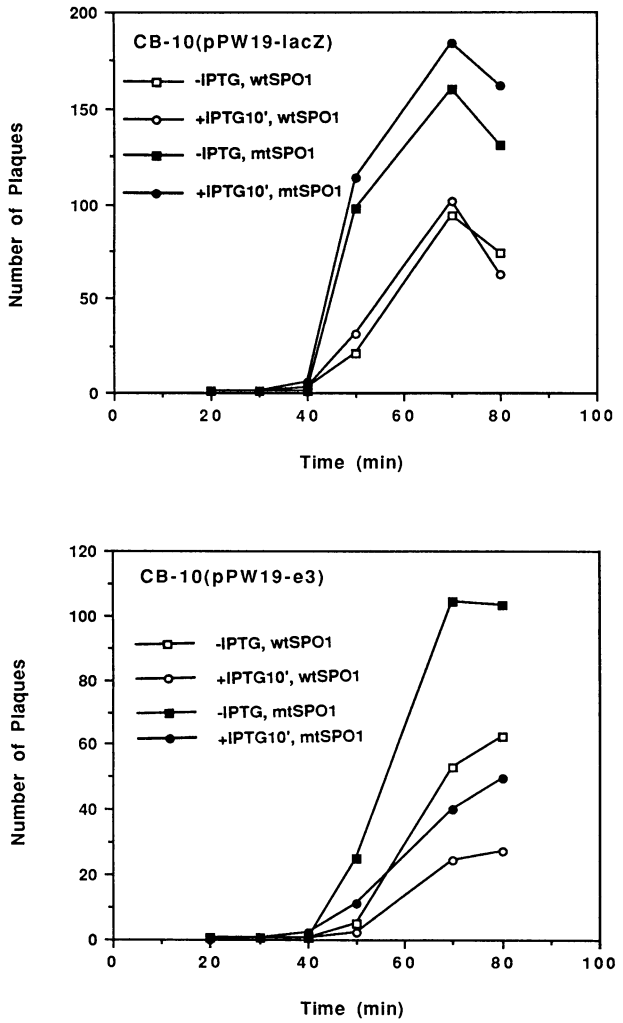

FIG. 11. Effects of expression of plasmid-borne *e3* gene on progeny phage production. Cultures of CB-10(pPW19-e3) or CB-10(pPW19-lacZ) were infected with wild-type SPO1 (open symbols) or mutant $e3^{m3}$ (closed symbols), and single-step growth experiments were performed as described in the legend to Fig. 10. Cultures in which expression of the plasmid-borne *e3* gene was induced by addition of IPTG at 10 min before infection are represented by circles. Cultures without IPTG are represented by squares.

DNA synthesis takes place in the absence of the middle gene-specific sigma (our unpublished results; to the best of our knowledge, shutoff of host gene action has not been tested in this respect). (iii) There are few other obvious functions for the early genes to perform. Except for gene *28*, all genes known to be involved in replication or morphogenesis are known to be expressed at middle or late times, known to be transcribed from middle or late promoters, and/or known to be located in regions of middle or late transcription (58). (iv) Several restriction fragments, representing different regions of the terminal redundancy, were unclonable (6).

We have now shown that the product of one of these genes, *e3*, does indeed have host shutoff activity. When *e3* was expressed, in either *B. subtilis* or *E. coli*, cell growth was inhibited, the incorporation of precursors into DNA, RNA, and protein was inhibited, and colony formation was prevented. (It is not surprising that a gene from a *B. subtilis* phage could shut off *E. coli* functions, since many functions in the two bacteria are accomplished by homologous molecules utilizing similar mechanisms. For examples, see various chapters of *Bacillus subtilis and Other Gram-Positive Bacteria* [55]). The primary target of the E3-induced shutoff effect remains to be identified. The long time required for the decrease in DNA, RNA, and protein synthesis makes it plausible that any or all of these effects is an indirect result of the primary effect of E3. (It is even conceivable that one or another of the decreases in isotope incorporation is an indirect result of an E3-induced inhibition of the incorporation of isotope into the effective precursor pool.) However, directly or indirectly, E3 is capable of shutting off processes that are shut off during infection.

The fact that shutoff can be caused by *e3* expression in uninfected cells is not sufficient to show that E3 is responsible for the shutoff that occurs during SPO1 infection. Proteins such as the products of T4 gene *32* or lambda gene *P*, which have other well-defined roles in phage multiplication, are toxic to cells for reasons that are presumably incidental to their intended function (27, 35), and this could be true of E3 as well. The shutoff that occurred during infection was not prevented by an *e3* nonsense mutation, and it occurred more rapidly than that caused by expression of the *e3* gene in uninfected cells. Thus, if E3 plays a role in host shutoff during infection, it must be only one component of the host shutoff machinery, which must include elements whose function is redundant to that of E3.

We think it likely that a substantial number of gene products are, in fact, involved in host shutoff, both for the reasons cited above with regard to the SPO1 early genes and because that would be consistent with patterns seen for other virulent phages. T7 has at least 6 and T4 has at least 10 genes whose products have been shown specifically to inactivate or destroy particular host functions and/or structures (17, 29, 34), and each has many other genes whose expression is lethal to the host but whose mechanisms of action are unknown (29, 60). Some of these genes have apparently redundant functions. For example, most of the 10 T4 gene products mentioned above have activities capable of inhibiting host transcription, either by inactivating RNA polymerase or by destroying template, but mutations that inactivate several of these genes have no effect on the shutoff of transcription during infection. Most strikingly, the *alt* and *mod* gene products each cause ADP ribosylation of the same amino acid in the α subunit of RNA polymerase, inhibiting its transcription activity as measured in vitro, but strains with both of those genes mutated still shut off host transcription normally (17, 19, 29).

The duplicated cluster of SPO1 early genes, with many highly active early promoters, and at least for the two genes that are now known, apparently efficient ribosome binding sites, appears designed to produce large quantities of gene products as soon as possible after infection, a quality desirable for host shutoff functions, which quickly must stop macromolecular syntheses that are occurring at thousands of sites in the cell. Again, this is consistent with observations of other phages, which express shutoff functions from highly active immediate early promoters (10, 29, 34, 60). The fact that shutoff would not, a priori, be essential for production of at least some progeny phage, plus the possible redundancy of shutoff functions, offers a ready explanation for the failure to find any conditional lethal mutations in the early gene region of the terminal redundancy (44, 58).

Negative effect of E3 on phage multiplication. Both of the genes studied here, and SPO1 early genes in general (13, 15, 63), were themselves shut off after a brief period of activity. The reason for this may be the same as the reason for the

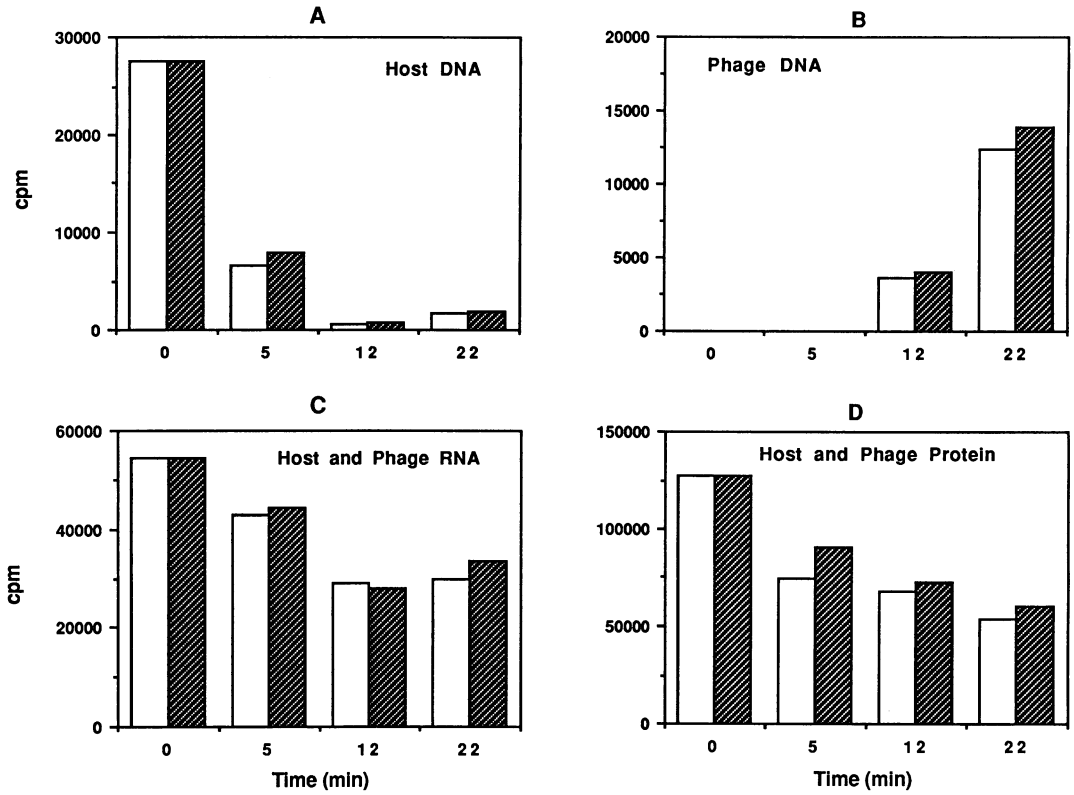

FIG. 12. Effect of the *e3* mutation on macromolecular synthesis during infection. CB-10 cells, infected at an MOI of about 5.0 with wild-type SPO1 (open bars) or SPO1 $e3^{m3}$ (striped bars), were pulse-labeled just before infection (time 0) and at 5, 12, and 22 minutes after infection, and the newly synthesized macromolecules were measured as described in Materials and Methods. The small differences between mutant and wild type that are seen in this figure were not observed consistently. Since phage DNA synthesis does not begin until about 10 min after infection, earlier time points are not reported in that curve. Significant counts from [5-^{3}H]uridine were incorporated into DNA late in infection (presumably having been converted to cytosine). This did not happen in uninfected cells or early in infection and was presumably a result of changes in nucleotide metabolism caused by infection. Thus, it is likely that the 22-min data in panel C include a significant amount of DNA and thus represent an overestimate of RNA synthesis at that point.

shutoff of host genes: to keep them from competing with the phage-productive middle and late genes. Another reason may be that their shutoff activities are not 100% specific to host functions, so that if they are present for too long or in too large quantities, they can be toxic to phage development as well. This possibility was suggested by the fact that, at high multiplicities of infection, the *e3* nonsense mutant produced a larger burst than did wild-type SPO1.

The activity of the *e3* gene product thus caused a selective disadvantage in high multiplicity infections. The continued existence of such a gene argues that high multiplicities are rare in nature and/or that some natural conditions and host strains provide a greater demand for *e3* function than was seen in our laboratory conditions.

ACKNOWLEDGMENTS

We thank Caroline Lee for making plasmid pCL2, Maurie Mintz for making pMM3, Angela Tobias for making pAT3-anti, and Yibin Wang for advice and assistance with several experiments.

This work was supported by research grant DMB-8801703 from the National Science Foundation.

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