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A host-shutoff early gene of *Bacillus subtilis* bacteriophage SPO1

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A HOST-SHUTOFF EARLY GENE OF
BACILLUS SUBTILIS BACTERIOPHAGE SPO1

by

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OF THE REQUIREMENT FOR THE DEGREE
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ABSTRACT

A Host-shutoff Early Gene of *Bacillus subtilis*
Bacteriophage SPO1

by

Ping Wei

Shutoff of host biosynthesis is one of the earliest and most dramatic events occurring in viral infection and requires the expression of viral early genes. To understand the mechanisms of bacteriophage SPO1 induced host-shutoff, two SPO1 early genes, e3 and e22, were cloned and sequenced, and the roles of e3 in host-shutoff and in phage growth were studied.

Both e3 and e22 are novel genes, and are actively expressed during the first few minutes of infection before being promptly shut off. Expression of a plasmid-borne e3 gene, in either *B. subtilis* or *E. coli*, caused the inhibition of host DNA, RNA and protein synthesis, and ultimately led to cell death. To identify the primary target of e3-induced shutoff, an e3-resistant *E. coli* mutant was isolated and analyzed. Plasmid libraries of this mutant's genomic DNA, when screened for genes that could protect wild-type *E. coli* against e3, yielded the *rpoB* and *dksA* genes, which specify the RNA polymerase β subunit and a suppressor for DnaK, respectively. The wild-type dksA gene, but not the wild-type rpoB gene, was able to protect against e3, suggesting that the primary e3-resistant mutation was in the rpoB gene and that protection by the dksA gene depended upon overexpression from the plasmid. I suggest that e3 acts by distorting the structure of the host RNA polymerase, thus
preventing host transcription, and that this distortion can be prevented or reversed by a chaperonin-like activity specified by \( \textit{dksA} \).

The host-shutoff still occurred normally during infection by an SPO1 mutant which lacked \( e3 \) activity, and it occurred much more rapidly than that caused by expression of \( e3 \) in uninfected cells. Thus, the \( e3 \) product must be only one component of the host-shutoff machinery, which must include elements whose function is redundant to that of \( e3 \). At high multiplicities of infection, the mutant SPO1 produced more phage progeny than the wild-type SPO1, suggesting that high concentrations of \( e3 \) can be inhibitory to phage growth as well as to host function. Perhaps for that reason, expression of both \( e3 \) and \( e22 \) is shut off after a brief period of high activity.
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Chapter 1. Introduction

I. General Introduction to Host Shutoff Action in Viral Infection.

Viruses are obligate intracellular parasites of their hosts. They are generally classified as animal, plant and bacterial viruses (the last group is often called bacteriophage) depending on their host. In spite of their simple form (most viruses are composed of a protein coat and a nucleic acid core) and relatively small genome sizes (from a few hundred nucleotides to a few hundred kilobases), most virulent viruses have the striking ability of taking over the host metabolic machinery to redirect it to the synthesis of predominantly viral components. This is achieved by selectively shutting off host DNA, RNA and protein syntheses, and replacing them almost entirely with viral syntheses. The shutoff of host macromolecular synthesis is an active process which requires the expression of viral genes. It usually is accomplished early in infection, before the replication of the viral genome and the synthesis of viral structural proteins. The obvious outcomes of viral infection are production of viral progeny and killing of the host (for comprehensive reviews, see Fraenkel-Conrat and Wagner, 1984; Calendar, 1988; Levine, 1992).

Although the shutoff of host macromolecular synthesis is one of the earliest and most dramatic events occurring in viral infection, there is only limited knowledge about its mechanisms and the viral genes involved in this process. It has become increasingly clear that there is no single underlying principle by which viruses shut off host biosynthesis. Rather, different viruses utilize different mechanisms, and various viral and host factors are
involved. This introduction will discuss briefly the shutoff action caused by various animal and bacterial virulent viruses and will focus mainly on the shutoff mechanisms of the best known viruses.

A. Mechanisms of Host Shutoff in Animal Virus Infection.

Most virulent animal viruses that cause cytopathic effects on permissive hosts inhibit host macromolecular synthesis upon infection. Depending on the type of viruses and the cells they infect, the extent of inhibition varies. The temporal pattern of the inhibition also differs from virus to virus. For example, herpes simplex virus, vaccinia virus (both double-stranded DNA viruses), and vesicular stomatitis virus (a single-stranded RNA virus) shut off host DNA, RNA and protein syntheses concomitantly in the early phase of infection. Poliovirus and mengovirus (both single-stranded RNA viruses) do not drastically affect host DNA synthesis until the shutoff of host RNA and protein syntheses has been completed (within two hours of the seven-hour infection cycle), suggesting that the effect on DNA synthesis is a secondary result of one or more other inhibitions. Adenoviruses types 2 and 5 (double-stranded DNA viruses) shut off host DNA synthesis in the early phase of viral infection, whereas the shutoff of host RNA and protein synthesis occurs eight to ten hours later, in the late phase of viral infection (for reviews, see Fraenkel-Conrat and Wagner, 1984). Of the various macromolecular changes induced by cytocidal viruses, the mechanisms for inhibition of host protein synthesis have been most thoroughly studied, and most of the results were obtained from studies on poliovirus and adenovirus infections. Hardly any studies
have been pursued to understand the mechanisms for inhibition of host DNA or RNA synthesis.


It is commonly believed that both poliovirus and adenovirus shut off host protein synthesis by inactivating the eukaryotic translation initiation factors, eIF-4F and eIF-2 (for reviews, see Sonenberg, 1990; Thach, 1992). Other possibilities, such as viral infection changing the stability and/or translatability of cellular mRNAs, or viral RNA outcompeting the cellular mRNAs, which could explain the same outcome, are apparently invalid. Untranslated cellular mRNAs are structurally and functionally intact, and the shutoff still takes place in cells infected with mutant viruses which no longer synthesize viral RNA or protein (Fernandez-Munoz and Darnell, 1976; Kaufmann et al., 1976; Babich et al., 1983; reviewed by Schneider and Shenk, 1987).

Translation initiation factors and their modification by viruses.
As Figure 1 shows, eIF-2 and eIF-4F participate in two critical steps in the process of eukaryotic translation initiation. eIF-2 consists of three subunits, α, β and γ. In the presence of GTP, eIF-2 will bind to initiator tRNA, Met-tRNA, and form a ternary complex. This complex then binds to the 40S ribosomal subunit to generate a 43S preinitiation complex. eIF-4F also consists of three subunits: eIF-4A', eIF-4E and p220. eIF-4A' is a 50 kd ATP-dependent RNA helicase which unwinds 5' secondary structure in the mRNA untranslated region; eIF-4E (also called cap-binding protein, CBP) is a 25 kd protein that binds to the mRNA 5' cap structure, and its activity is
Figure 1. Formation of mRNA ribosome complexes and recycling of eIF-2. This figure and legends are quoted from Sonenberg, 1990. "The figure illustrates (i) the requirement for eIF-2 in ternary complex formation. This complex transfers the initiator Met-tRNA (Met-tRNA_f) to 40S ribosome; (ii) the requirement for eIF-4A, -4B, and -4F, and for ATP hydrolysis for mRNA binding to the 43S preinitiation complex; and (iii) the requirement for recycling of eIF-2 by GEF (guanine nucleotide exchange factor). For clarity, other initiation factors have been omitted."
phosphorylation dependent; p220 is a 220 kd protein whose integrity is required for eIF-4F function. eIF-4F, together with eIF-4A, 4B, and in the presence of ATP, will bind to a 5’ capped-mRNA and transfer it to the 43S preinitiation complex to form an mRNA-ribosome complex. eIF-2 is then released from this complex and recycled by guanine nucleotide exchange factor (GEF, also called eIF-2B) in the presence of GTP (reviewed by Moldave, 1985; Pain, 1986).

Poliovirus inactivates eIF-4F by proteolysis of the p220 subunit (Sonenberg, 1987), and adenovirus does so by dephosphorylation of the eIF-4E subunit (Hung and Schneider, 1991). Both processes result in most of the cellular mRNAs being prevented from binding to ribosomes, and, therefore, prevent their translation. Inactivation of eIF-4F is not sufficient for complete shutoff of host protein synthesis in poliovirus infection (Bonneau and Sonenberg, 1987). Poliovirus and adenovirus ensure the complete inhibition of host protein synthesis by inactivating some of the eIF-2 factor in cells through phosphorylation of its α subunit. Phosphorylated eIF-2α traps the GEF and neither can be recycled any more (Safer, 1983; O’Neill and Racaniello, 1989; O’Malley et al., 1989).

**Mechanisms for viral RNA translation independent of eIF-4F.**

Poliovirus and adenovirus have evolved specific mechanisms for discriminating cellular mRNAs from viral RNAs, so that viral RNA translation will not be affected by the inactivated eIF-4F factor. Unlike most eukaryotic cellular mRNAs and viral RNAs, poliovirus RNA is uncapped (Hewlett et al., 1976; Nomoto et al., 1976) and has a long 5’ untranslated region (5’ UTR, about 750 nucleotides) (Racaniello and Baltimore, 1981). Ribosomes can bind directly to an internal site in the 5’-UTR and proceed
with translation in the absence of initiation factor eIF-4F (Pelletier and Sonenberg, 1988; reviewed by Jackson et al., 1990). This mechanism of translation initiation is also used by another picornavirus, encephalomyocarditis virus (Jang et al., 1988, 1989).

Adenovirus shuts off host protein synthesis in the late phase of infection when late viral RNA is prominent (Ginsberg, 1969). All the late RNAs of adenovirus contain a common 200-nucleotide 5' noncoding region, called the tripartite leader (Logan and Shenk, 1984). This region enables adenovirus late RNAs to be translated independent of eIF-4F, presumably facilitated by an unstructured sequence at the 5' end of the tripartite leader (Dolph et al., 1988, 1990). This mechanism of translation distinguishes late adenovirus RNAs from cellular mRNAs, and presumably from early viral RNA, too, since early viral RNAs lack the tripartite leader (Huang and Schneider, 1991).

Mechanisms for control of eIF-2α phosphorylation and its role in shutoff of host protein synthesis. An increase in eIF-2α phosphorylation occurs relatively late in viral infection and only to a moderate extent. In uninfected cells, 5-10% of the eIF-2α is phosphorylated. In poliovirus infected cells, phosphorylation of eIF-2α increases approximately three-fold at three to five hours postinfection, when most host shutoff is completed (Black et al., 1989; O’Neill and Racaniello, 1989). In adenovirus-infected cells, phosphorylation of eIF-2α increases to 25-30% late in infection, and a mutant adenovirus (Ad5-dl331) which fails to produce a small viral RNA, VA1, phosphorylates approximately 90% of eIF-2α (O’Malley et al., 1989; Duncan, 1990). The kinase that phosphorylates the α subunit of eIF-2 is a 68 kd, double-stranded RNA-activated kinase, called p68 kinase or DAI
(Black et al., 1989). It has been proposed that the accumulated untranslated cellular mRNAs and large quantities of viral RNAs made late in infection constitute an amount of double-stranded RNA sufficient to trigger the activation of the latent p68 kinase. It, in turn, phosphorylates the eIF-2α. However, the level of this phosphorylation is controlled, so that it is high enough for the complete shutoff of host protein synthesis, but not sufficient for inhibition of viral RNA translation (Black et al., 1989; Sonenberg, 1990; Duncan, 1990). It has been shown that phosphorylation of about 30% of eIF-2α in rabbit reticulocytes and HeLa cells results in complete inhibition of protein synthesis, but in adenovirus infected cells only the translation of host and early viral RNAs is inhibited (O’Malley et al., 1989).

To maintain the proper level of eIF-2α phosphorylation, viruses have evolved mechanisms that specifically regulate the p68 kinase activity. In poliovirus infected cells, the steady-state level of the p68 kinase is reduced dramatically late in infection, possibly due to a viral-induced degradation (Black et al., 1989). In adenovirus infected cells, a small viral RNA, VA₁, can bind directly to p68 kinase and sequester it from binding to double-stranded RNA, thus preventing the massive inhibition of protein synthesis (Katze et al., 1987; Galabru et al., 1989). VA₁ is synthesized in large amount late in adenovirus infection and is required for efficient translation of late viral RNA. Infection with Ad5-dl331, a VA₁-deletion mutant, results in global extinction of translation (Thimmappaya et al., 1982; Reichel et al., 1985; Schneider et al., 1985).

**Viral factors.** Viral factors that are responsible for the modification of translation initiation factors are still under intensive study. Genetic evidence indicates that poliovirus protease 2A mediates the cleavage of p220
(Bernstein et al., 1985; Krausslich et al., 1987; O'Neill and Racaniello, 1989). However, protease 2A does not cleave p220 directly; rather, an unidentified cellular protease that is activated by 2A does (Lloyd et al., 1986; Krausslich et al., 1987). Sonenberg speculated that protease 2A is also responsible for the degradation of p68 kinase (Sonenberg, 1990), but no evidence has yet been provided. No viral factor that dephosphorylates eIF-4E has been identified.

2. Possible Mechanisms for Inhibition of Host Protein Synthesis Utilized by Other Animal Viruses.

Since proteolysis of p220 and phosphorylation of eIF-2α were not detected in a large number of viruses (reviewed by Duncan, 1990), and studies on the dephosphorylation of eIF-4E were only carried out in adenovirus (Huang and Schneider, 1991), it is possible that viruses other than poliovirus and adenovirus have evolved different host shutoff mechanisms, and dephosphorylation of eIF-4E may also apply to other viral systems. In fact, RNA stability, RNA transport and RNA competition seem to play important roles in some viral shutoff systems (reviewed by Schneider and Shenk, 1987; Katze and Agy, 1990). It was found that herpes simplex virus, vaccinia virus, reovirus, encephalomyocarditis (EMC) virus and human immunodeficiency virus (HIV) induce cellular mRNA and/or rRNA degradation. Influenza virus and adenovirus block newly synthesized mRNAs from entering the cytoplasm (Katze and Krug, 1990), although in adenovirus, this process is not sufficient for the host shutoff (Huang and Schneider, 1990). Viral RNAs of mengovirus, EMC virus and reovirus have both structural and quantitative advantages over the cellular mRNAs. These
viruses probably inhibit host protein synthesis by RNA competition (Jen and Thach, 1982; Lemay, 1988; Schneider and Shenk, 1987). The nature of these mechanisms and the viral factors involved are unknown.

B. Mechanisms for Host Shutoff in Bacteriophage Infection.

Most virulent bacteriophages cause shutoff of host DNA, RNA and protein syntheses shortly after infection [some small filamentous phages, like M13, are exceptional. They continuously produce phage progeny which are extruded from the cell membrane, without affecting host growth as long as sufficient nutrients are available (reviewed by Model and Russel, 1988)]. Bacteriophages have a relatively shorter lytic cycle than animal viruses (12 minutes to less than one hour vs. a few hours to a few days at 37°C), and the shutoff usually is completed within a few minutes (see Calendar, 1988). For example, *E. coli* phage T4 has an infection cycle of about 25 minutes at 30°C, and it shuts off approximately 80% of host RNA synthesis in the first two minutes of infection. At five minutes postinfection, at which time T4 DNA replication starts, the inhibition is almost complete (Snustad and Bursch, 1977; Tigges et al., 1977).

Unlike animal viruses, for which the mechanisms for shutoff of host protein synthesis are best known, bacteriophages are better understood for genes involved in the shutoff of host DNA and RNA syntheses. Fairly little is known about genes responsible for the shutoff of host protein synthesis, or about mechanisms by which these shutoffs take place. Most of the studies on host shutoff by bacteriophages were carried out on *E. coli* phages, especially T4 (reviewed by Snustad et al., 1983; Kutter et al., 1993). T4 shuts off host DNA, RNA and protein syntheses concomitantly in the first
few minutes of infection. The shutoff of host DNA synthesis appears to be independent of the shutoff of host RNA synthesis, since a viral mutation affecting host DNA shutoff had no detectable effect on RNA shutoff and vice versa. T4, like all other T phages, has the ability to degrade the host cell genome and reutilizes the degradation products for phage synthesis. However, host shutoff is independent of host chromosome degradation, since T4 mutants deficient in chromosome degradation shut off host macromolecular synthesis normally (Snustad and Conroy, 1974; Snustad et al., 1976a; 1976b; Snustad and Bursch, 1977; Tigges et al., 1977; reviewed by Snustad et al., 1983).

1. Genes Involved in the Shutoff of Host DNA Synthesis.

Gene *ndd* of T4. In T4 infected cells, host DNA synthesis ceases at about four minutes after infection at 30°C (Snustad et al., 1976a). Concomitantly, host nucleoid undergoes a gross rearrangement: the host DNA moves from a largely central region of the cell to the periphery and the ribosomes migrate to the center of the cell. This process is called nuclear disruption, and is mediated by a T4 gene, *ndd* (Snustad and Conroy, 1974). Infection with a nuclear disruption deficient phage, *ndd*<sup>-</sup>, shows no detectable difference from normal infection except for a delay in host DNA shutoff, which then takes place at about ten minutes after infection (Snustad et al., 1976a; Snustad and Bursch, 1977). These results indicate that the *ndd* gene product is involved in the inhibition of host DNA replication (reviewed by Snustad et al., 1983; Kutter et al., 1993). However, *ndd* is not an essential gene. An *ndd*-defective T4 yielded the same burst size and growth rate as wild-type T4 (Snustad and Conroy, 1974). The shutoff that takes
place in \textit{ndd}· phage infection can not be explained by the onset of host DNA
degradation, which starts at about ten minutes postinfection, since a
degradation-deficient and \textit{ndd}· double mutant still caused the delayed
shutoff, indicating an independent and redundant activity of host shutoff.
The viral gene specifying this activity is unidentified (Snustad et al., 1976a).

The \textit{ndd} gene is actively expressed early in T4 infection, reaching its
maximum transcription rate at three to six minutes after infection and being
shut off by about 12 minutes postinfection. The \textit{ndd} gene encodes a small,
basic protein that is synthesized in large quantities. More than 4,000
molecules of Ndd protein are produced per infected cell, and are possibly
associated with the host cell membrane(Koerner et al., 1979; Chapman et al.,
1988). It has been proposed that the Ndd protein binds to the host
chromosome at multiple sites and attaches it to the cell membrane (reviewed
by Snustad et al., 1983; Kutter et al., 1993). The mechanisms by which the
Ndd protein selectively binds to host chromosome and shuts off host DNA
replication are still unknown.

\textbf{Gene A* of \textit{\Phi}X174.} Gene A* of coliphage \textit{\Phi}X174 is responsible for
the shutoff of host DNA synthesis, and is possibly involved in the inhibition
of transcription and translation, too (reviewed by Hayashi et al., 1988). \textit{E. coli}
cells infected with \textit{\Phi}X174 showed clear repression of host DNA
synthesis (Lindqvist and Sinsheimer, 1967). Expression of gene A* from a
multi-copy plasmid resulted in inhibition of DNA synthesis and cell division
(Colasanti and Denhaart, 1985). \textit{\Phi}X174 also inhibits the synthesis of several
catalytic enzymes to various extents, whereas a mutant \textit{\Phi}X174 with an
amber mutation in gene A* gave no such repression. The inhibition of
protein synthesis was suggested to be effected at the transcriptional level
(Pal and Poddar, 1980; Ghosh et al., 1985). Protein A* binds to single-stranded as well as double-stranded DNA, and possesses an endonuclease activity (Eisenberg and Ascarelli, 1981). It was hypothesized that protein A* blocks DNA replication by binding to replicating DNA and breaking DNA at the growing forks or by making a physical obstruction which prevents the replication machinery from binding to DNA (Colasanti and Denharft, 1985). The actual mechanisms by which A* protein shuts off host DNA synthesis and its role in the regulation of host transcription and translation still await elucidation.

2. Genes Involved in the Shutoff of Host RNA Synthesis.

Genes \textit{alt}, \textit{mod}, and \textit{alc} of T4. T4 shuts off host RNA synthesis by at least two mechanisms: (1) ADP-ribosylation of the host RNA polymerase by the \textit{alt} and \textit{mod} gene products; and (2) inhibition of transcription of cytosine-containing DNA by the \textit{alc} gene product (reviewed by Snustad et al., 1983; Kutter et al., 1993).

T4 utilizes the host RNA polymerase throughout its infection cycle (Schachner and Zillig, 1971). However, the host RNA polymerase is modified at different times after infection by various phage factors to facilitate the expression of T4 genes (reviewed by Rabussay, 1983). The first modification is ADP-ribosylation of the RNA polymerase \( \alpha \) subunits. The Alt protein, injected together with the T4 genome, ADP-ribosylates one of the \( \alpha \) subunits immediately after infection. The Mod protein, specified by an early gene, ADP-ribosylates both of the \( \alpha \) subunits a few minutes later (Horvitz, 1974a; 1974b; Rohrer et al., 1975). \textit{In vitro} studies showed that within one minute of infection, the Alt protein alone reduced RNA
polymerase activity by 40%. In the absence of the alc gene product, the Alt and Mod proteins reduced the RNA polymerase activity by 50% at five minutes postinfection (Drivdahl and Kutter, 1990). This reduction in RNA polymerase activity is mainly with respect to the host DNA and some of the immediate-early genes but not to the majority of the T4 DNA (Mailhammer et al., 1975; Goldfarb, 1981), indicating that ADP-ribosylation of the RNA polymerase plays an important role in shutting off the host transcription and phage immediate-early gene transcription. However, mutants defective in both alt and mod had no detectable effect on phage growth and showed normal shutoff of some host transcription (Goff and Setzer, 1980), suggesting that ADP-ribosylation is not the sole mechanism for host shutoff.

T4 also shuts off host transcription by taking advantage of the fact that T4 contains an unusual base. The genomes of all T-even phages contain glucosylated hydroxymethylcytosine instead of cytosine (reviewed by Mattews, 1977; Mattews and Allen, 1983). T4 directs the synthesis of a dCTPase, which excludes the incorporation of cytosine into the phage genome. Two phage-specified nucleases, endonuclease II and IV, ensure this by cleaving any cytosine-containing DNA. A triple mutant of T4, [dCTPase-, endo II-, endo IV-], allowed the synthesis of cytosine-containing phage DNA, but no phage was produced (Kutter et al., 1975). It was found that this mutant phage did not synthesize phage late proteins due to the inhibition of transcription of late genes (Wu and Geiduschek, 1975). These results indicate that T4 has the ability to inhibit transcription of cytosine-containing DNA. A fourth mutation allowed the synthesis of phage late proteins and produced phages containing almost 100% cytosine. This mutation was mapped to a gene, called alc (Snyder at al., 1976). In vivo and
*in vitro* studies demonstrate that the Alc protein is a general inhibitor to transcription of cytosine-containing DNA (Pearson and Snyder, 1980; Kutter et al., 1981; Drivdahl and Kutter, 1990). This property of Alc provides another mechanism for the shutoff of host RNA synthesis, since normally only the host DNA contains cytosine (reviewed by Snustad et al., 1983; Kutter et al., 1993).

Genetic studies have shown that the Alc protein functions by interacting with the RNA polymerase (a mutation in the RNA polymerase β subunit permitted the above triple mutant to propagate cytosine T4 even if the phage are genotypically *alc*+ (Snyder and Jorissen, 1988; Kutter et al., 1993), although *in vitro* studies indicated that Alc proteins only bind loosely to the RNA polymerase (Drivdahl and Kutter, 1990). The *alc* gene is active early in infection. *In vitro* studies showed that the Alc protein alone reduced the RNA polymerase activity by 25-30% in the first five minutes of infection, and it inhibits transcription at the elongation but not the initiation phase (Kutter et al., 1984; Drivdahl and Kutter, 1990). Recently, the *alc* gene was cloned, and *in vitro* studies from Kashlev et al. (Kutter et al., 1993) indicate that under physiological conditions and in the absence of any other factors, the Alc protein interacts with the elongation complex and releases transcripts at specific stop sites. These sites have a consensus sequence of CCCUGAAA.

**Genes 0.7 and 2 of T7.** T7, like many other phages, organizes its genes topologically according to their functions, and their expression is under tight sequential controls (reviewed by Hausmann, 1988). T7 early genes, located at the left end of the T7 genome, are injected first into host cells and transcribed by host RNA polymerase. Some of the early proteins
then function in preparing the intracellular environment for efficient viral production, including shutting off host macromolecular synthesis. One of the early genes encodes a phage-specific RNA polymerase which then transcribes phage middle genes (located to the right of the early genes and functioning in phage replication) and late genes (located on the right half of the phage genome and encoding phage morphological functions).

T7 infection shuts off host RNA synthesis by five to seven minutes after infection in the absence of any middle gene transcription. It has been demonstrated that an early gene, gene 0.7, is responsible for this shutoff (Brunovski and Summers, 1971, 1972; Rothman-Denes et al., 1973). The 0.7 gene product has a protein kinase activity which phosphorylates a number of proteins, including the β’ subunit of the host RNA polymerase (Rahmsdorf et al., 1974; Zillig et al., 1975; Robertson and Nicholson, 1990). Since T7 encodes its own RNA polymerase, inactivation of the host RNA polymerase would readily explain the shutoff of host transcription. It has been proposed that the phosphorylation of the host RNA polymerase inactivates its activity, and thereby inhibits host transcription (Geiduschek and Kassavetis, 1988; Hausmann, 1988). However, phosphorylation of the host RNA polymerase β’ subunit only reduced its activity by about 30% (Hesselbach and Nakada, 1977). Moreover, a 0.7 gene mutant which did not exhibit kinase activity in vivo, still catalyzed the host shutoff efficiently (Rothman-Denes et al., 1973). Therefore, the β’ phosphorylation is not the only cause of host shutoff. Recently, it was found that the kinase and host shutoff activities of gene 0.7 are two independent functions, and are exerted by separate protein domains (Michalewicz and Nicholson, 1992). How the
host shutoff domain of the gene 0.7 protein inhibits host RNA synthesis is still unknown.

Gene 2 of T7, also encodes an inhibitor of the host RNA polymerase. It produces a small protein which binds to the host RNA polymerase and reduces its activity by about 80% (Hesselbach and Nakada, 1977). However, gene 2 is a middle gene, and its expression depends on the synthesis of T7 early proteins, particularly the synthesis of phage RNA polymerase. Therefore, its inhibitory function on the host RNA polymerase must occur later than that of the gene 0.7 product. The gene 2 product is also involved in phage DNA replication and phage packaging, and thus may be more important for phage late functions than for host shutoff (Hesselbach and Nakada, 1977; Geiduschek and Kassavetis, 1988; Hausmann, 1988). The apparent redundancy of gene 0.7 and gene 2 functions could explain the fact that gene 0.7 is nonessential for phage growth.

T3 is a close relative of T7 and also encodes its own RNA polymerase and has gene 0.7 and gene 2. It is possible that T3 uses the same mechanisms for the shutoff of host transcription (reviewed by Kruger and Schroeder, 1981).

II. BACTERIOPHAGE SPO1 AS A SYSTEM FOR THE STUDY OF HOST SHUTOFF MECHANISMS.

Most studies on the shutoff of host macromolecular synthesis by bacteriophages were carried out in coliphage-infection systems, and very little is known about host shutoff mechanisms for phages that infect other bacteria. *Bacillus subtilis* is an endo-spore forming, gram-positive bacterium, and is probably the best studied bacterium other than *E. coli*
(Sonenshein et al., 1993). SPO1 is one of its large, virulent DNA phages and is one of the most extensively studied of the B. subtilis phages. SPO1 has many properties that are remarkably similar to coliphage T4: (1) similar morphologies: both phages have head and tail structures, and their tails have a contractile sheath and complex baseplate; (2) genome sizes: both are among the largest of bacteriophages (142 kb genome for SPO1, 166 kb for T4); (3) unusual base substitution in their genomes: SPO1 has hydroxymethyluracil completely replacing thymine, and T4 uses hydroxymethylcytosine instead of cytosine; (4) gene regulation: both phages regulate their gene expression by sequential modification of host RNA polymerase, allowing the temporal appearance of different sets of gene products; (5) introns in genes: both phages contain self-splicing group I introns, one in SPO1, three in T4; the intron of SPO1 and the two of T4’s all occur in genes affecting DNA synthesis; (6) shutoff actions: both inhibit host macromolecular synthesis soon after infection, making them extremely virulent phages (reviewed by Stewart, 1993; Mosig and Eiserling, 1988). However, there are major differences between them with respect to the mechanisms by which shutoff is accomplished. For instance, SPO1 does not cause degradation of host DNA, and transcription of certain host genes continues throughout infection, so identification of which genes should be shut off must be based on more subtle distinctions than the presence or absence of the unusual base. Thus, study of SPO1 will illuminate distinctive mechanisms by which shutoff takes place, as well as providing insights into the evolutionary divergence of bacteriophages of gram-positive and gram-negative bacteria.
A. General Introduction to Bacteriophage SPO1.

A cascade of sigma factors and three classes of genes. SPO1 genes have been categorized as early, middle, and late, on the basis of the time when transcription begins (Gage and Geiduschek, 1971a; Talkington and Pero, 1977; Pero et al., 1979). As Figure 2 shows, SPO1 early genes are turned on shortly after infection and are transcribed by the host RNA polymerase, with the host major sigma factor A (Talkington and Pero, 1977; Moran, 1989). Early genes can be further divided into two subclasses, e and em, depending on their modes of shutoff. Genes of subclass e are only expressed for a very short period of time (from about one minute until about six to seven minutes postinfection), and their shutoff occurs before the onset of phage replication (at about ten minutes postinfection). Genes of subclass em are turned on at about the same time as e genes, but are turned off at the end of middle time (12-15 minutes after infection) (Gage and Geiduschek, 1971a; Heintz and Shub, 1982). Middle genes are transcribed, beginning at about five minutes after infection, by the host RNA polymerase that has been modified by a phage early gene product, gp28. Gene 28 specifies a middle gene-specific sigma factor (Duffy et al., 1975; Fox et al., 1976; Costanzo and Pero, 1983). Middle genes also have two subclasses, m and m1. Genes of subclass m are shut off at the end of middle time, whereas m1 genes continue to be expressed until the end of infection (about 28-30 minutes) (Gage and Geiduschek, 1971a; Heintz and Shub, 1982). Late genes are transcribed by the host RNA polymerase that has been modified by two middle gene products, gp33 and gp34. Gene 34 specifies a late gene sigma factor and the gene 33 product is also required for late gene transcription (Fox, 1976; Tjian and Pero, 1976; Costanzo et al., 1984; Gribskov and
Figure 2. Programs for SPO1 gene expression and development. The temporal expression patterns and sigma factors required for the transcription of SPO1 early (e and em), middle (m and m₁) and late (m₂₁ and l) genes are shown in the upper portion of the figure. The timing and major events during SPO1 development are shown below. The optimum lytic cycle of SPO1 infection at 37°C takes about 33 minutes. The figure and legend are modified from Gage and Geiduschek, 1971a.
Burgess, 1986). Late genes also can be separated into two groups, m2l and l. Genes of group m2l start transcription a little earlier than phage DNA synthesis, whereas expression of l genes apparently depends on the synthesis of phage DNA. Both m2l and l genes are active till the end of infection (Gage and Geiduschek, 1971a; Heintz and Shub, 1982). With this sequential modification of the host RNA polymerase by different sigma factors, SPO1 regulates the turn-on of its three major classes of genes (reviewed by Losick and Pero, 1981; Stewart, 1993).

Each sigma factor recognizes a different set of promoters, called early, middle and late promoters, respectively (their consensus sequences around the -10 and -35 regions are shown at the top of the next page). As expected, early gene promoters have the same -10 and -35 consensus sequences as most host genes (Talkington and Pero, 1978; Lee and Pero, 1981; Romeo et al., 1981).

<table>
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<th>Type of promoter</th>
<th>-35 Region</th>
<th>-10 Region</th>
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<tr>
<td>Early</td>
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<td>AT'AAAT'</td>
</tr>
<tr>
<td>Middle</td>
<td>AGGA--A-T</td>
<td>TTT'-TTT'</td>
</tr>
<tr>
<td>Late</td>
<td>CGTT'AGA</td>
<td>GAT'ATT'</td>
</tr>
</tbody>
</table>

Dashes in sequences represent nucleotide positions with no consensus. T' represents hydroxymethyluracil. This table is reproduced from Stewart, 1993.

Locations and functions of genes. SPO1 early, middle and late genes tend to be clustered, with most of the early genes located in a terminal redundancy region [12.4 kb direct repeats at the ends of the SPO1 genome (Kallen et al., 1962; Cregg and Stewart, 1978; Pero et al., 1979)], most of the
middle genes in the right half of the unique region, and most of the late
genes in the left half (Talkington and Pero, 1977; Pero et al, 1979). Genes
with related functions tend to be clustered, too. Of 39 genes that have been
identified and mapped by means of conditional lethal mutations, genes 35
and 1-3 function in virion assembly, 4-6 in head formation, 7-20 in tail
formation, 21-23 and 27-32 in DNA synthesis, and 33 and 34 in gene
regulation (Fig. 3) (Okubo et al., 1972; Curran and Stewart, 1985a).
Although the predicted number of SPO1 genes is 140 (Stewart, 1993), only
about 60 of them were identified as bands on polyacrylamide gel of protein
extracts of infected cells. These were distributed about equally among early,
middle and late genes, on the basis of their dependence on gp28, 33 and 34
(Reeve et al., 1978; Heintz and Shub, 1982).

Twenty-one early proteins, identified as mentioned above, were
named e1 through e21 in order of decreasing molecular weight (Heitz and
Shub, 1982). Most early transcription takes place within the 12.4 kb
terminal redundancy, each copy of which includes at least 13 early
promoters, most of which are highly active (Pero et al, 1979; Romeo et al,
1981; Brennan et al, 1981). These early promoters, 12 early genes, and
transcription terminators have been mapped to approximate locations in the
terminal redundancy (Brennan et al, 1981; Brennan and Geiduschek, 1983;
Romeo et al, 1981; Perkus and Shub, 1985) (Fig. 3). Early gene 28, and
three relatively weak early promoters are located outside the terminal
redundancy, in a region containing mostly middle genes (Chelm et al.,
1981). Except for gene 28, which encodes the sigma factor for middle gene
transcription, functions for SPO1 early genes are unknown.

Many of the middle gene products are enzymes for phage DNA
Figure 3. Genetic and restriction maps of the SPO1 genome and detailed map of the SPO1 terminal redundancy. *Top*. Genetic map of SPO1. The positions of the 39 SPO1 genes (indicated by numbers), which have been identified by means of conditional lethal mutations, are shown. *Center*. EcoRI* restriction map of SPO1. Numbers represent the 26 EcoRI* fragments. Arrows above the map indicate the locations of most of the SPO1 early (e), middle (m) and late (l) genes. *Bottom*. SPO1 terminal redundancy. EcoRI* fragments 10, 15, 20 and 26 are separated by bars above the map. Boxes, arrows and bars below the map represent the approximate positions of SPO1 early genes (eN), early promoters(PeN) and terminators(HN or LN). Maps are adopted from Stewart, 1993.
replication (reviewed by Stewart, 1993). These include a phage-specific DNA polymerase (Yehle and Ganesan, 1972; DeAntoni et al., 1985; Goodrich-Blair et al., 1990), enzymes for the synthesis of hydroxymethyl dUTP, including dCMP deaminase (Roscoe and Tucker, 1966; Okubo et al., 1972), dUMP hydroxymethylase (Roscoe and Tucker, 1966; Alegria et al., 1968), hydroxymethyl dUMP kinase (Kahan, 1971), dTTPase-dUTPase (Roscoe, 1969b; Price et al., 1972; Dunhan and Price, 1974), and dTMPase (Aposhia and Tremblay, 1966). The later two enzymes plus an inhibitor of thymidylate synthetase (Haslam et al., 1967) are also important in preventing thymidine incorporation into the phage genome and the maintenance of a dTTP pool. Except for the hydroxymethyl dUMP kinase, whose activity was not determined, the activities for these enzymes were first detected at about six to ten minutes after infection, after which time they increased quickly, reaching a maximum at about 25 minutes after infection (Yehle and Ganesan, 1972; Roscoe and Tucker, 1966; Alegria et al., 1968; Roscoe, 1969a; Dunhan and Price, 1974). DNA polymerase, dUMP hydroxymethylase and hydroxymethyl dUMP kinase are specified by genes 31, 29 and 23, respectively (reviewed by Stewart, 1993).

SPO1 late genes, as with many phages, specify proteins for phage structural components and assembly. Fifty-three proteins were identified in phage particles, including 28 in the baseplate, six in the sheath and tube, three in the neck, and sixteen in the head (Parker and Eiserling, 1983). Genes for most of these proteins have not been mapped (for reviews, see Hemphill and Whitely, 1975; Stewart, 1993).
B. Shutoff of Host Macromolecular Synthesis by SPO1.

SPO1 inhibits host DNA, RNA and protein syntheses shortly after infection (reviewed by Stewart, 1993). Host DNA synthesis ceases by about five to seven minutes after infection (Shub, 1966; Wilson and Gage, 1971; Yehle and Ganesan, 1972). This shutoff requires the transcription of phage early gene(s) (Gage and Geiduschek, 1971b), and is independent of phage middle gene expression or the modification of host RNA polymerase, since shutoff takes place in sus28 (deficient in gp28 synthesis) infection (our lab unpublished results). Unlike T4, SPO1 infection does not cause degradation of the host genome (Roscoe, 1969a; Yehle and Ganesan, 1972). Evidence indicates that the phage-specified dTTPase-dUTPase is not required for this shutoff. dTTPase-dUTPase is a middle gene product. Its enzymatic activity was first detected at six to seven minutes after infection (Roscoe, 1969b; Dunhan and Price, 1974), by which time significant shutoff of host DNA synthesis had occurred. Even at the time of its maximal activity, at 25 minutes after infection, only about 15% of the dTTP in the nucleotide pool was degraded (Yehle and Ganesan, 1972). Moreover, a dTTPase-dUTPase deficient mutant of φe, which is a close relative of SPO1, shut off host replication normally (Roscoe, 1969b). The inhibitor of thymidylate synthetase is also not required for shutoff, since a mutant bacterial host, in which the requirement for thymidylate synthetase was circumvented, still had its DNA synthesis shut off by φe infection (Roscoe, 1969b).

Host RNA and protein syntheses are dramatically reduced between two and eight minutes of SPO1 infection (reviewed by Stewart, 1993). SPO1 has the ability to discriminate not only between host and phage RNAs, but also between different host RNAs. Most host mRNA synthesis was
almost completely shut off by eight minutes postinfection, whereas the synthesis of host rRNA continued (Shub, 1966; Gage and Geiduschk, 1971a; Webb and Spiegelman, 1984). Infection does prevent the normal increase in the rate of rRNA synthesis in response to a shift to enriched media, does not occur during SPO1 infection (Webb and Spiegelman, 1984). Nearly all host protein synthesis, as detected by SDS-PAGE electrophoresis of pulse-labelled proteins, had stopped by seven to eight minutes after infection (Reeve et al., 1978; Heintz and Shub, 1982). The shutoff rate of host protein synthesis is comparable to that caused by actinomycin in uninfected cells (Shub, 1966), suggesting that the shutoff of host protein synthesis may be solely the result of the inhibition of host transcription.

None of the SPO1 genes that are responsible for the shutoff of host macromolecular synthesis has been identified, nor have the mechanisms controlling these shutoffs been explored.

C. Specific Research Projects

I hypothesize that many of the SPO1 early genes are involved in host shutoff, for the following reasons: (1) Host shutoff begins too early to be accounted for by middle gene activity, and significant shutoff effects have occurred before the middle genes become active (Shub, 1966; Yehle and Ganesan, 1972; Gage and Geiduschk, 1971a,b; Heintz and Shub, 1982; Wei and Stewart, 1993). (2) The shutoff of host DNA synthesis takes place in the absence of the middle gene-specific sigma factor (our unpublished results). To the best of our knowledge, shutoff of host gene action has not been tested in this respect. (3) 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 either 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 (Stewart, 1993). (4) The selective advantage obtained from the rapid shutoff of host biosynthesis, which would otherwise compete with the corresponding phage syntheses, offers a plausible explanation for the evolution of the highly active duplicated cluster of early genes. (5) Most restriction fragments from the terminal redundancy were unclonable (Curran and Stewart, 1985a).

To identify SPO1 genes that are responsible for host shutoff and understand the mechanisms by which the shutoff takes place, I have begun an analysis of the SPO1 early genes in the terminal redundancy. I chose to start with early gene \( e_3 \), because it occupies most of EcoRI* fragment 26, which is one of the unclonable fragments from the terminal redundancy (Curran and Stewart, 1985a; Perkus and Shub, 1985). In the course of this analysis, I discovered another gene just upstream of \( e_3 \), overlapping EcoRI* fragments 15 and 26, which we named \( e_{22} \). I present the nucleotide sequences and expression pattern of genes \( e_3 \) and \( e_{22} \), and show that the \( e_3 \) product does cause the inhibition of host DNA, RNA and protein synthesis, and ultimately cell death. By analysis of mutants resistant to the cell killing effects of \( e_3 \), I show that \( e_3 \) probably acts by distorting the structure of the host RNA polymerase. However, a strain of SPO1 carrying an \( e_3 \) nonsense mutation is not deficient in host shutoff or phage production, suggesting redundancy of the \( e_3 \) function.
Chapter 2. Materials and Methods

Bacterial and Phage Strains.

Bacterial and bacteriophage strains used in this work are listed in Table 1.

Media and Growth Conditions.

Trypticase Soy Agar (Becton Dickinson) was used for top and bottom agar at 12 g/l and 40 g/l, respectively. Tryptose blood agar base (Difco) was used at 15.4 g/l for top agar from which plaque lifts were made. Liquid broth media included NY (8 g/l nutrient broth, 5 g/l yeast extract (Difco)) and VY (Stewart et al., 1971). Defined liquid media included: adsorption buffer (0.05 M Tris, pH 7.5; 0.1 M NaCl; 0.01 M MgSO4); Spizizen's salts medium (Spizizen, 1958); C1 (Spizizen's salts medium plus glucose plus complete amino acids) (Stewart, 1969); 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 (Ap) was used at 50 μg/ml; chloramphenicol (Cm) at 10 μg/ml for *E. coli* and 5 μg/ml for *B. subtilis*; kanamycin (Km) at 50 μg/ml; tetracycline (Tc) at 30 μg/ml. Isopropyl β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 0.2 mM (48 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) at 50 μg/ml.

SPO1 lysates were prepared by infecting mid-log phase *B. subtilis* cells (2x10⁸ cells/ml) at a multiplicity of 4-6 and shaking at 37°C until lysis. Cell debris was removed by centrifugation for 10 min at 8000 g. The average titer was 1-2x10¹⁰ PFU/ml. For concentration and/or
Table 1. Bacterial and Bacteriophage Strains

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<th>Strain</th>
<th>Relevant Genotype or Description</th>
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<td>CB-10</td>
<td><em>his</em>&lt;sup&gt;−&lt;/sup&gt; <em>trp</em>&lt;sup&gt;−&lt;/sup&gt; <em>sup</em>&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>CB-313</td>
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<td>71-18</td>
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<td>Sambrook et al, 1989</td>
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<td></td>
<td>F&lt;sup&gt;+&lt;/sup&gt;[*proAB&lt;sup&gt;+&lt;/sup&gt; lac&lt;sup&gt;l&lt;/sup&gt; lacZ&lt;sup&gt;ΔM15&lt;/sup&gt;]</td>
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</tr>
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<td><em>dut1 ung1 thl-1 relA1 / pCJ105(Cm&lt;sup&gt;F&lt;/sup&gt; F&lt;sup&gt;+&lt;/sup&gt;)</em></td>
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<td>MC4100</td>
<td>Δ(<em>argF&lt;sup&gt;−&lt;/sup&gt;</em>-<em>lac</em>) <em>rpsL150 (Str&lt;sup&gt;F&lt;/sup&gt;) relA1</em></td>
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<td><strong>Bacteriophages</strong></td>
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<td>SPO&lt;sub&gt;e3&lt;/sub&gt;&lt;sup&gt;m3&lt;/sup&gt;</td>
<td>A mutant SPO1 having a nonsense mutation at the codon 3 of e3 gene.</td>
<td>This work</td>
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removal from broth medium, lysates were centrifuged for 150 min at
13,000 g, and phage pellets were resuspended in adsorption buffer at 4°C overnight (avoiding vortexing or vigorous shaking).

Oligonucleotide Primers.

Oligonucleotide primers used for sequencing and polymerase chain reaction (PCR), and oligonucleotide linkers used for modifying different cloning vectors are listed in Table 2. Oligonucleotides synthesized in this department were purified by heating the samples at 55°C for 5 h, allowing ammonia to evaporate overnight in a hood, and desalting using Econo-
Pac™ 10DG columns (Bio-Rad) according to the manufacturer's instructions.

Plasmids.

pDH32 and its derivatives.

See Figure 4 for map and description of pDH32.

pCL2 has a spontaneously derived clonable mutant of SPO1 EcoRI* fragment 26 (Lee and Pero, 1980a, b), which Caroline Lee inserted at the EcoRI site of pDH32.

pPW110 is a modification of pDH32, in which the polylinker, just upstream of the lacZ gene, has been replaced by a new polylinker: EcoRI-SstI-KpnI-BglII-HindIII-SnaB1-BamH1. This linker exchange was accomplished in three consecutive steps: (1) inserting a SnaBI-linker at the SmaI site of pUC19; (2) inserting the EcoRI-SacI-KpnI-SnaBI-BamHI linker (ESKSB-linker) of the modified pUC19 in place of the EcoRI-SmaI-BamHI linker of pDH32; (3) creating two new sites in the ESKSB-linker by
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<td>5' AGAGGAAGAGGACATCC 3'</td>
<td>935-951</td>
</tr>
<tr>
<td>G</td>
<td>5' ATACGGTTGTGATGGTCG 3'</td>
<td>1243-1226</td>
</tr>
<tr>
<td>H</td>
<td>5' AGTCTAAAGTACGCTGAG 3'</td>
<td>1312-1328</td>
</tr>
<tr>
<td>I</td>
<td>5' ACGCCCTAGAACCCTTAGC 3'</td>
<td>835-820</td>
</tr>
<tr>
<td>LYS3AAA</td>
<td>5' AAATGGCTAAATCAAATAAA 3'</td>
<td>742-760</td>
</tr>
<tr>
<td>LYS3UAG$^c$</td>
<td>5' AAATGGCTTAGTCAAATAAA 3'</td>
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<td>MUTLYS3$^c$</td>
<td>5' AGATTTCAATGGCTAGTCA</td>
<td>735-767</td>
</tr>
<tr>
<td></td>
<td>AATAACGTGTAC 3'</td>
<td></td>
</tr>
<tr>
<td>pDH32-L</td>
<td>5' AAGGGTAACGTATTGCCG 3'</td>
<td>3864-3848$^d$</td>
</tr>
<tr>
<td>pDH32-R</td>
<td>5' CTTCCACAGTAGTCACC 3'</td>
<td>3751-3768$^d$</td>
</tr>
<tr>
<td>CLA-L</td>
<td>5' TGGCAACGCTACCTTTCG 3'</td>
<td>2108-2091$^e$</td>
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<tr>
<td>CLA-R</td>
<td>5' GTATAAATGGGCTCGCG 3'</td>
<td>1996-2012$^e$</td>
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<tr>
<td>SnaBI linker</td>
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<td></td>
<td>3' CATGCATG 5'</td>
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<tr>
<td>KBHS linker$^f$</td>
<td>5' CAGATCTAAGCTTAC 3'</td>
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<td>3' CATGGTCTAGATTGGAATG 5'</td>
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<tr>
<td>HSBS linker$^g$</td>
<td>5' AGCTTATGATCCCCGGAGATCTG 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3' ATACTAGGCCCCTCTAGACAGCT 5'</td>
<td></td>
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</table>

$^a$Oligonucleotide Primers

$^b$Position in the sequence

$^c$Mutagenic oligonucleotide

$^d$Range of positions

$^e$Range of positions

$^f$ kbHS linker

$^g$kbHS linker
a. Except for primers E and LYS3UAG which were purchased from American Synthesis Inc. and Oligo Etc. Inc., respectively, all other oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer by K. Muthukrishnan, J. Nichols, or R. Brantley in this department.

b. Numbers, unless otherwise labelled, refer to the positions of these primers in the 1.5 kb sequenced region (see Chapter 3).

c. Underlined sequences are the mismatch sites in the mutagenic oligo, MUTLYS3, used for mutagenesis; and in the screening oligo, LYS3UAG, used for identifying the mutant SPO1 (see later part of this chapter).

d. Numbers are the positions in plasmid pDH32.

e. Numbers are the positions in plasmid pACYC177.

f. KBHS linker represents the KpnI-BglII-HindIII-SnaBI linker, used for generating plasmid pPW110.

g. HSBS linker represents the HindIII-SmaI-BglII-SalI linker, used for generating plasmid pPW19.
Figure 4. Map of the *B. subtilis* integration vector pDH32.
pDH32 was provided by D. Henner. It is a modification of the *B. subtilis*
integration vector ptrpBG1, in which the *B. subtilis* amyE gene was split
into two parts, front (amyE F) and back (amyE B), allowing the region in
between them to be integrated into the *B. subtilis* chromosome by
homologous recombination (Shimotsu and Henner, 1986). This region
contains a promoterless *E. coli lacZ* gene in translational fusion to the
ribosome-binding site of *B. subtilis* gene *SpoVG* (Zuber and Losick, 1983),
and a chloramphenicol acetyl transferase gene (CAT) for selection
(Horinouchi and Weisblum, 1982). The plasmid also has a β-lactamase
gene (Ap) and a pBR322 replication origin from pBR322 (Bolivar et al.,
1977). Transcription direction of genes is shown by arrow. Positions of
some of the restriction enzyme-cleavage sites are also shown. The
poly linker in plasmid pPW110 is shown to the right of that of pDH32.
inserting a KpnI-BglII-HindIII-SnaBI linker between the KpnI and SnaBI sites of the modified pDH32.

**pPW19 and its derivatives.**

pPW19 is described in Figure 5.

pPW19-e3, pPW19-e3\textsuperscript{m3}, and pPW19-e3\textsuperscript{m71} have the promoterless wild-type e3 gene, or the e3 gene with nonsense mutations in codon 3 or 71, respectively, inserted at the SmaI site of pPW19, oriented such that they are expressed correctly from the Spac-I promoter. The e3 and e3\textsuperscript{m3} genes were provided on the 890 bp PCR fragment shown in Chapter 3, made from wild-type or e3\textsuperscript{m3} mutant SPO1 DNA, respectively. The cloned fragment carrying the e3\textsuperscript{m71} gene (940 bp) was made by PCR from pCL2, using the same left-end primer used for e3 and e3\textsuperscript{m3}, but with primer pDH32-R (Table 2) as the right-end primer, whose sequence is 70-51 bp upstream of the EcoRI site on pDH32 vector (Fig. 4).

pPW19-lacZ was formed by inserting, between the HindIII and SmaI sites of pPW19, a 4.3 kb HindIII-ScaI fragment of pPW110 carrying the promoterless lacZ gene, oriented such that the lacX gene was correctly expressed from the Spac-I promoter.

**pUC and pEMBL derivatives.**

pUC19, pEMBL19(+), pUC118, and pUC119 were all described by Sambrook et al. (1989). The later three are phagemids used for generating single-stranded versions of cloned fragments.

The above 940-bp PCR fragment of e3\textsuperscript{m71} from pCL2 was cleaved with EcoRI and cloned between the SmaI and EcoRI sites of pUC118, forming pPW13, which produces the transcribed strand of the e3 gene as
Figure 5. Map of the *E. coli / B. subtilis* expression vector pPW19. pPW19 was formed by introducing a new polylinker, HindIII-SmaI-BglII-SalI, between the HindIII and SalI sites of pSI-1, an *E. coli/B. subtilis* shuttle vector provided by D. Henner. It has the IPTG-inducible Spac-I promoter, a hybrid of SPO1 promoter Pe5 and the *E. coli* lactose operator (Yansura and Henner, 1984), 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 *B. licheniformis* penicillinase gene; replication origins from pBR322 (Bolivar et al., 1977) and pUB110 (McKenzie et al., 1987), permitting replication in either *E. coli* or *B. subtilis*; and the chloramphenicol acetyl transferase gene from pC194 (Horinouchi and Weisblum, 1982), providing chloramphenicol-resistance. pPW19 and its derivatives show structural instability (Bron et al., 1991; Janniere et al., 1990), so it was 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.
single-stranded DNA.

pPW22wt has the 890 bp promoterless wild-type e3 gene (same as that in pPW19-e3), cloned into the SmaI site of pUC19, such that the non-transcribed strand of the e3 gene will be transcribed by the lac promoter. pAT3anti has the EcoRI fragment of pPW22wt carrying the e3 gene, cloned into the EcoRI site of pEMBL19(+), such that the transcribed strand of the e3 gene is produced as single-stranded DNA. pMS1 is a pUC19-derivative, E. coli/B. subtilis shuttle vector, obtained from E. P. Geiduschek (Sayre and Geiduschek, 1988).

pACYC177 and its derivatives.

pACYC177 is described in Figure 6.

pPW40, pPW50 and pPW60 (see Chapter 5) contain approximately 8.8 kb, 7.3 kb and 2.05 kb, respectively, fragments of HpaII partial-digested DH5R chromosomal DNA, cloned into the ClaI site of pACYC177. These plasmids have fragments cloned in such a way that the genes on them could be correctly expressed from the vector's kanamycin promoter.

pPW70 contains an approximately 4.1 kb fragment of HpaII-partial digested DH5 chromosomal DNA, cloned into the ClaI site of pACYC177. The sites on the E. coli chromosome, from which the insert fragments originated, are determined in Chapter 5.

Derived from pPW50, pPW51 has a 5.4 kb HindIII fragment, carrying the rplL' and rpoB' genes, cloned into the HindIII site of pACYC177; pPW52 has a 2.5 kb PvuII fragment, carrying the genes rplJ' and rplL' and their promoter, P'L10, cloned into the SmaI site of pACYC177; pPW53 has a 3.2 kb ClaI-SmaI fragment, carrying the disrupted rpoB' gene, cloned between the ClaI and SmaI sites of
Figure 6. *E. coli* cloning vector pACYC177. pACYC177 (Chang and Cohen, 1978; Rose, 1988) is a low copy-number (~20 copy/cell) plasmid. It carries the origin of replication from plasmid p15A, enabling it to co-exist with vectors having the ColE1 origin (eg. pBR322, pUC19). It also has two antibiotic selection markers, the β-lactamase gene (Ap) from Tn3 and the kanamycin-resistant gene (Km) from Tn903. Transcription direction of genes is shown by arrow. Some of the unique restriction enzyme sites used in cloning are also shown.
pACYC177. pPW54 has a 4.1 kb BamHI-SacI fragment of pRL385 (from R. Landick, Landick and Stewart, 1990), carrying the wild-type promoterless rpoB gene, filled in with T4 DNA polymerase and cloned into the SmaI site of pACYC177.

Derived from pPW60, pPW61 has a 1.2 kb PvuII fragment, carrying the sfsA' gene and its promoter; pPW62 has an 1.3 kb HincII-EcoRV fragment, carrying the dksA' gene and its promoter; and pPW64 has a 0.5 kb PvuII-EcoRV fragment, carrying the disrupted dksA' gene, each cloned into the SmaI site of pACYC177; pPW63 has a 1.0 kb ClaI-EcoRV fragment from pPW60, carrying the promoterless dksA' gene, cloned between the ClaI and SmaI sites of pACYC177. pPW63wt and pPW63mt each has a 1.1 kb ClaI-Bst1107I fragment from plasmids pJK538 and pJK543 (provided by E. Craig), respectively, cloned between the ClaI and SmaI sites of pACYC177. pJK538 contains the wild-type dksA gene and pJK543 has a mutant dksA gene, in which the NdeI site in the dksA gene has been filled in by Klenow fragment (Kang and Craig, 1990).

The cloned fragments in these plasmids are in the same orientation as those of their parental plasmids, pPW50 and pPW60, such that the kanamycin promoter correctly expresses genes on the fragments. Plasmids, which have the same inserts cloned in the opposite orientation, were named pPW52R, pPW54R, pPW61R, pPW62R.

**DNA Manipulation and Transformation.**

Routine procedures for preparation and enzymatic manipulation of plasmid and SPO1 genomic DNA, and for transformation of *B. subtilis* or *E. coli*, were performed as described by Stewart (1969), Curran and
Stewart (1985a) and Sambrook et al. (1989, p. 1.21-1.41 and p. 1.53-1.84). Single-stranded plasmid DNA was prepared using the M13KO7 helper phage/MV1184 host cell system (Vieira and Messing, 1987) as described by Sambrook et al. (1989, p. 4.44-4.50). Double-stranded plasmid DNA for sequencing was prepared as described in the Promega Protocols and Applications Guide (Promega). DNA bands were recovered from agarose gels using the Prep-a-Gene DNA Purification Matrix Kit (Bio-Rad) following the manufacturer’s instructions.

**Polymerase Chain Reaction.**

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

**DNA Sequencing and Sequencing Analysis.**

Single- and double-stranded DNA sequencing were performed by the dideoxy chain termination method (Sanger et al., 1977), using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp.). Direct sequencing of purified PCR fragments was performed as
described by Wang et al. (1990). Each sequence was determined at least
twice, from both strands. Sequence analysis used the software package
provided by the Genetics Computer Group (GCG) at the University of
Wisconsin, and the Genbank, EMBL, and SwissProt databases. G values
for the ribosome-binding sites were calculated using the parameters of
Tinoco et al. (1973). with procedures exemplified by Rabinowitz and
colleagues (McLaughlin et al., 1981; Murray and Rabinowitz, 1982).

**Liquid Hybridization of Pulse-labeled RNAs.**

*B. subtilis* CB-10 cells, grown in NY medium at 37°C to
approximately 2x10⁸ cells/ml, were infected with SPO1 at a MOI of 4-6.
At various times after infection, aliquots were pulse-labeled for 2 min with
40 μCi/ml of 5,6-³H uridine (52 Ci/mM, ICN) and stopped by adding ice-
cold NaN₃ (from Sigma) to 25 mM. RNA was isolated by the sonication
method of Gilman et al. as described by Ausubel et al. (1989, p. 4.4.1-
4.4.4). The newly synthesized e3 RNA was measured by a modification of
the liquid hybridization procedure of Gage and Geiduschek (1971a). Four
micrograms of RNA from the pulse-labeled culture were incubated with an
excess amount (10 μg) of single-stranded e3 DNA (complementary to e3
RNA, produced from pPW13) in 400 μl of 2xSSC (1xSSC is 0.15 M NaCl
and 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 15
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 μ nitrocellulose filter (from
Millipore). The filter was washed with 30 ml of 0.3% TCA in 0.1x SSC,
dried, and counted with Liquifluor scintillation fluid (NEN Research
Products). Non-specific hybridization (less than 2% of the highest e3-specific signal) was measured by hybridizing with 10 µg of single-stranded pUC119 DNA and subtracted from the amount of e3-specific hybridization. The total RNA input in each hybridization reaction (about 10⁵-10⁶ cpm) was measured by incubating the RNA in the same condition except no single-stranded DNA was added, and then precipitated the RNA directly with 5% TCA without prior RNase treatment.

**RNA preparation and Northern blot analysis.**

RNA was isolated from *B. subtilis* cells using a modification of the procedure of Curran and Stewart (1985b). CB-10 cells, grown at 37°C to approximately 2x10⁸ cells/ml, were infected with SPO1 at a MOI of 4-6. At various times after infection, 1.5 ml aliquots were transferred to microfuge tubes containing cold NaN₃, producing a final NaN₃ concentration of 25 mM. The cells were centrifuged and resuspended in 50 µl of RNA Isolation Buffer I (15 mM Tri-HCl, pH 8.0, 15% sucrose, 8 mM EDTA) plus 10 mg/ml lysozyme, and incubated at 37°C for 15 min. Three microliters of diethyl pyrocarbonate (DEPC, from Sigma) and 50 µl of RNA Isolation Buffer II (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM sodium citrate, 1.5% SDS) were added and the mixture was incubated at 37°C for 5 more min. It was then chilled on ice for 2 min, and 25 µl of H₂O saturated with NaCl were added. The mixture was kept at -20°C for 10 min before centrifuging at 12 K rpm for 10 min. The supernatant was extracted once with an equal volume of sevag mix (24:1 chloroform : isoamyl alcohol) 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. (1989). Five micrograms 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/ml of ethidium bromide 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 using a Stratalinker™ UV Crosslinker following the manufacturer's instructions (Stratagene, La Jolla, CA). Hybridization probes used were the 890 and 297 bp PCR fragments (Chapter 3) for e3 and e22, respectively. They were labeled with 32P-ATP (5000 μCi/mM, New England Nuclear), using a Random Primer DNA Labelling Kit (Boehringer Mannheim) following the manufacturer's instructions. The specific activities of the probes were about 10^9 cpm/μg. The filters were prehybridized for 4 hours at 60°C in (10X Denhardt’s, 50 mM Na3PO4, 0.5% SDS, 0.2X SSC). Approximately 0.1 μg denatured probe was added, and the hybridization proceeded overnight at 60°C. The filters were washed twice in 2X SSC for 10 minutes at room temperature, and twice in 0.2X SSC, 0.1% SDS for 20 minutes at 60°C.

Protein Labeling and Electrophoresis.

CB-10 cells, grown in C6 medium at 37°C to approximately 1x10^8 cells/ml, were infected, at 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/ml of
\(^{35}\text{S}-\text{Met/Cys}\) (Amersham, 1140 Ci/mM). Alternatively, CB-10 (pPW19-e3) cells, were pulse-labeled after various times of IPTG induction. Cells were lysed and proteins extracted as described by Heintz and Shub (1982). 10 \(\mu\text{l}\) of each protein extract were loaded on a sodium dodecyl sulfate-12% polyacrylamide gel (SDS-PAGE) and separated by electrophoresis as described by Sambrook et al. (1989). 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 was carried out using the Muta-Gene Phagemid \textit{in vitro} Mutagenesis Kit (Bio-Rad), following the manufacturer's instructions. Phagemid pAT3anti was used to prepare the uracil-containing single-stranded DNA template, and the 33-mer primer MUTLYS3 was used as the mutagenic oligonucleotide (see Table 2). The mutation converts codon 3 of the e3 gene, AAA, into the nonsense codon TAG. This result was 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 (1988). An EcoRI fragment of the mutant pAT3anti, carrying the mutant e3\(^{m3}\) gene, was cloned into the EcoRI site of pMS1, an \textit{E. coli}:\textit{B. subtilis} shuttle vector. This recombinant plasmid was transformed into CB-313, a \textit{B. subtilis} suppressor strain, which inserts lysine at UAA or UAG codons (Mulbry et al., 1989). These transformants,
growing in VY plus 5 μg/ml chloramphenicol, were infected by wild-type SPO1 at a multiplicity of about 1.0. Fifteen min later, the cultures were diluted 50 fold into VY and Cm. Fourty-five min later, 0.02 ml of CHCl₃ were added per ml of culture, shaking was continued for one more minute, and progeny phage were plated on a CB-313 lawn, using 15.4 g/l TBAB as top agar. Recombinants that had incorporated the e₃ᵐ₃ mutation were identified by filter hybridization. Duplicate plaque-lift filters were made (using BA85 filters, Schleicher and Schuell) and probed by mutant (LYS3UAG) and wild-type (LYS3AAA) oligonucleotides. The oligonucleotides were end-labeled with γ⁻³²P-ATP (Amersham, 4500 Ci/mM) and purified as described by Sambrook et al. (1989), yielding a specific activity of 7 to 10x10⁹ cpm/μg. Five filters, each containing approximately 1200 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 2x10⁶ cpm/ml of the appropriate oligonucleotide probe, at 42°C for 16 h. Filters were washed twice with 100 ml of 6xSSC at room temperature for 5 min (low stringency) and then washed individually in 300 ml 6x SSC in a 400-ml beaker at 38°C for 2 min (high stringency). To obtain recombinants that had the e₃ᵐ₃ mutation in both copies of the TR, some of the plaques that hybridized to both the wild-type and mutant probes were propagated through another round of infection of CB-313, and the progeny in which the e₃ᵐ₃ mutation had replaced the wild-type allele in both copies of the TR were identified by the same filter hybridization procedures. This mutant SPO1 was named SPO1e₃ᵐ₃.
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, 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/NY. In all cases, cells were grown at 37°C to approximately $1 \times 10^8$ cells/ml, pulse-labeling was for three minutes, with 0.8 μCi/ml of the appropriate radioactive precursor in 0.5 ml aliquots of the culture being tested, and the time shown for each was the beginning of the 3-minute period. Host DNA was labeled with 5-$^3$H thymidine (49 Ci/mMole, ICN); host and/or phage RNA with 5-$^3$H uridine (29 C/mMole, Amersham); and host and/or phage protein with 4,5-$^3$H leucine (80 Ci/mMole, ICN). Each pulse was terminated by precipitation with 5% TCA. Precipitates were collected on 0.45 μ nitrocellulose filters, which were washed, dried, and counted as above. For leucine incorporation, the TCA-precipitates were boiled for 15 minutes and then kept on ice for at least 10 min before filtration. Phage DNA was labeled with 5,6-$^3$H uridine (52 Ci/mMole, ICN). Each pulse was terminated by adding 0.5 ml cold 2.0 N KOH. The samples were incubated for 6-16 h at 37°C, neutralized with 0.5 ml 2.0 N HCl, and precipitated with TCA as above.
Isolation of e3-Resistant *E. coli* Strains.

Newly transformed DH5(pPW19-e3wt) cells were plated onto Cm (10 µg/ml) plus IPTG (0.2 mM) plates, and incubated at 37°C for 1-2 days. Clones that grew up on these plates were first tested for the integrity of their endogenous pPW19-e3wt plasmids: plasmids were isolated from each clone and digested with EcoRI and SnaBI, separately. Plasmids that showed the correct sizes of the EcoRI- and SnaBI-cleaved bands were then transformed into competent DH5 cells and plated on Cm plus IPTG plates to test their cell-killing ability upon IPTG-induction. Intact pPW19-e3 plasmids prevented colony formation on IPTG plates. Strains whose plasmid still behaved like pPW19-e3 were presumed to have e3-resistant mutations on their chromosomes, and were tested to verify that. They were cured of plasmid by extended growth without selection, and transformed with verified pPW19-e3, and plated on IPTG plates. Strains with e3-resistant mutations on the DH5 chromosome were able to form Cm^R^, IPTG^R^ colonies. One such resistant strain was named DH5^R^.

Isolation of *E. coli* Chromosomal DNA for Constructing Plasmid Libraries.

*E. coli* chromosomal DNA was prepared using a modified procedure of Ausubel et al. (1989, p. 2.4.1-2.4.2). A single colony of *E. coli* DH5 or DH5^R^ was inoculated into 100 ml NY in a 500 ml flask, and incubated with vigorous shaking at 37°C for 12 h. All subsequent steps required gentle but thorough mixture of the sample so as to minimize breakage of the chromosomal DNA and obtain the maximum yield. Cells were pelleted at 8000g for 5 min and resuspended in 37.4 ml of TE (10 mM Tris-HCl, pH
8.0, 1 mM EDTA, pH 8.0). Ten milliliters of lysozyme (Sigma) and 0.4 ml of 10 mg/ml RNase A were added, and the mixture was incubated at 37°C for 30 min before 2 ml of 10% SDS and 0.2 ml of 20 mg/ml of proteinase K (from Sigma, pretreated at 37°C for 30 min) were added, and the incubation was continued for another hour. Then, 6.7 ml of 5 M NaCl and 5.3 ml of CTAB/NaCl solution [10% hexadecyltrimethyl ammonium bromide (from Sigma) in 0.7 M NaCl] were added consecutively, and the mixture was heated at 65°C for 10 min before being extracted once with an equal volume of phenol/sevag solution (25 : 24 : 1 of phenol : chloroform : isoamyl alcohol). The sample was centrifuged at 12,000 g for 5 min and the supernatant was collected (with difficulty, due to the extreme viscosity of the sample. The white interphase was removed before pipetting the upper layer with trimmed blue pipetman tips). The collected sample was extracted again with an equal volume of sevag. A 0.6 V of isopropanol was added to the supernatant and a white stringy DNA formed. It was briefly pelleted (8000 g, 5 min) and washed with 20 ml of 70% ethanol and dried for 5 min under vacuum before being resuspended in 1 ml of TE overnight without vortexing. Five microliters of 10 mg/ml of RNase A were then added and the sample was incubated at 37°C for 1 h.

HpaII Partial-digestion of E. coli Chromosomal DNA.

150 μl of E. coli chromosomal DNA were pipetted using a trimmed yellow pipetman tip into a microfuge tube, and 10xL buffer (from Boehringer Mannheim, 100 mM Tris-HCl, pH7.5, 100 mM MgCl2, 10 mM Dithioerythritol) and 10 mg/ml of bovine serum albumin (BSA, from New England Biolabs) were added in to a final concentration of 1x and 100
μg/ml, respectively, in a total volume of 540 μl. The tube was placed on ice for 2-3 h with gentle mixture of the sample once every hour to achieve an uniform distribution of the DNA. Partially digested DNA whose sizes were distributed mostly between 2 and 4 kb was obtained by digesting 234 μl of this DNA sample with 26 μl of 0.05 unit/μl HpaII enzyme for 20 min. DNA whose sizes were between 4 and 10 kb was obtained by digesting the same amount of DNA with 26 μl of 0.025 unit/μl HpaII for 30 min. Digestion was stopped by adding 15 μl of 0.5 M EDTA. Samples were then separated on a 0.5% agarose gel at 30V for 4 h. DNA between 2-4 kb or 4-10 kb was cut from the gel and recovered as described above.

Constructing and Testing of E. coli Libraries.

200 ng of ClaI-cleaved, dephosphorylated pACYC177 plasmid was ligated with 100 ng of HpaII partially-digested E. coli genomic DNA under normal ligation conditions (Sambrook et al., 1989) in a total of 10 μl. The ligation sample was precipitated with 10 μg of tRNA, 0.3 M ammonium acetate and 2 V of ethanol at 13,000 g for 15 min. The pellet was washed with 60 μl of 70% ethanol, and centrifuged again as above before being dried and resuspended in 4 μl of 0.5 TE. This DNA was stored directly at -20°C as a plasmid library.

To test the integrity of the library, 1 μl of the library DNA was transformed into 20 μl of competent MC4100 cells by electroporation, and blue transformants on Ap plus X-gal plates were counted. Library DNAs which gave ratios of blue clones/total transformants greater than 1x10^-4 (indicating that less than 10,000 clones had any fragment of the E. coli
genomic DNA cloned) were used for further screening of e3-resistant clones.

**Screening of E. coli Libraries for e3-Resistant Clones.**

1 μl of library DNA was electroporated into 20 μl of competent DH5(pPW19-e3) cells and the transformation culture was plated on plates containing Ap (selecting for the pACYC177-derivative plasmid), Cm (selecting for the pPW19-e3 plasmid) and IPTG (selecting for e3-resistance). Clones that were ApR, CmR and IPTGR were tested to confirm that the e3-resistance was solely from the expression of the ApR plasmid: plasmid preparations from these clones (containing both the ApR and CmR plasmids) were transformed into competent DH5 cells. DH5(pACYC177-derivatives) and DH5(pPW19-e3) clones were then obtained by plating the transformation culture onto Ap and Cm plates separately, and confirmed by streaking transformants onto Cm and Ap plates, respectively. DH5(pPW19-e3) clones were also streaked onto Cm plus IPTG plates to test the integrity of the pPW19-e3 plasmid. ApR plasmids whose original strains still maintained the intact pPW19-e3 plasmid were retransformed into DH5(pPW19-e3). Plasmids expressing e3-resistant genes allowed these transformants to grow on Ap, Cm plus IPTG plates.

**Electroporation.**

Competent cells for electroporation were prepared as described (Hanahan et al., 1991). Electroporation was conducted using 0.2 cm Potter-type cuvettes and a Gene Pulser™ apparatus (Bio-Rad) under
conditions of 200 Ω resistance, 25 μF capacity, and 12.5 kV/cm field strength (Dower et al., 1988; Hanahan et al., 1991). Electroporated cells were added to 1 ml of SOC medium (Hanahan et al., 1991) and shaken at 37°C, 150 rpm for 1 h before being plated on selective plates.
Chapter 3. Cloning and Sequencing of SPO1
Early Genes e3 and e22

INTRODUCTION

The SPO1 terminal redundancy (TR) contains most of the highly active SPO1 early genes whose functions are unknown. All of the restriction fragments in the TR which were tested were refractory to cloning, and it was shown that this unclonability was not due to transcription from promoters on the cloned fragments reading through into the vector to cause expression of toxic vector gene(s), or to interfere with plasmid replication or transcription (Curran and Stewart, 1985a). Thus, it is likely that each of these fragments contains a gene or genes whose products are deleterious to cell growth, a trait that would be expected from host-shutoff genes. Thus, these unclonable fragments provide good candidates for studying genes that cause host shutoffs.

The three most intensely studied of these unclonable fragments are EcoRI* 15, 18 and 26 (Fig. 7). Fragment 26 is the smallest EcoRI* fragment of SPO1 (about 1.1 kb), and contains a strong early promoter, Pe5, and all of early gene, e3 (Lee et al., 1980b; Perkus and Shub, 1985). Fragment 18 has two relatively weak promoters, Pe1 and Pe1', which presumably transcribe gene e9. Fragment 15 includes at least four early genes and three strong early promoters, Pe2, Pe3 and Pe4. All three promoters are located on the right half of the fragment, with promoter Pe4 positioned about 0.4 kb upstream of promoter Pe5 (Lee and Pero, 1981; Romeo et al., 1981; Brennan et al., 1981; Perkus and Shub, 1985).

In this chapter, I describe the sequencing of fragment 26 and the
Figure 7. Cloning and sequencing strategies. (Top). Map of the TR. EcoRI* 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 (PeN) and the known early genes (eN), respectively. Arrows show the transcriptional direction of each promoter. (middle). Map of the 1.5 kb segment in the TR. Numbers 15, 26, and 10 above the map identify the relevant EcoRI* fragments. Arrows and boxes above the lines represent the positions of early promoters (PeN) and genes (eN), respectively. Numbers below the line indicate the positions, in the sequence shown in the next figure, at which the EcoRI* fragments and genes begin and end. (Bottom). Positions of primers used for PCR and sequencing, and the positions of cloned fragments. Arrows indicate the direction of replication. The 306-bp Sau3A fragment [fragment 15(1), nucleotides 1 to 306] contains promoter Pe4, the 156-bp AluI fragment [fragment 26(1), nucleotides 504 to 659] contains promoter Pe5, the 297-bp PCR fragments (nucleotides 281 to 577) was generated from primers A and B, and the 890-bp PCR fragment (nucleotides 642 to 1531) was amplified from primers D and E. Each cloned fragment was sequenced from at least two individual clones and in both orientations.
right end of fragment 15. For an earlier project, I had attempted to isolate all of the promoters from the TR. The results of that study, which included the isolation of promoters Pe4 and Pe5 and thus provided the foundation for the sequencing of genes e22 and e3, are described first.

RESULTS

Cloning and Sequencing of SPO1 Early Promoters in the Terminal Redundancy. SPO1 EcoRI* fragments 10, 15, 18 and 26, covering the terminal redundancy, were purified and cleaved with Alu I (for EcoRI*26) and Sau3A (for the others), and cloned into the polylinker of plasmid pPW110, just upstream of a promoterless lacZ gene. Promoter-containing clones were identified as blue colonies on X-gal plates, and the cloned fragments were sequenced using flanking primers pDH32-L and pDH32-R (see Table 2).

A dark blue clone from EcoRI*26, two very light blue clones from EcoRI*18, six dark blue clones from EcoRI*15, and six clones of varying darkness from EcoRI*10 were shown to contain small inserts, and their sequences were determined. These inserted fragments were named after their parental fragments [eg. 10(4) is cloned fragment number 4 from EcoRI*10]. Fragment 26(1) contains a 156-bp AluI fragment. All the six fragments from EcoRI*15 have a common 123-bp region [15(1) has the longest fragment of 306 bp]. Compared with published sequences of the regions immediately surrounding promoters Pe4 and Pe5 (Lee and Pero, 1981; Yansura and Henner, 1984; Greene et al., 1986), it became evident that fragment 26(1) contains promoter Pe5, and all six clones from EcoRI*15 have promoter Pe4. My sequences of Pe4 and Pe5 differ
slightly from those previous determinations, which also differ slightly from each other. Each of Pe4 and Pe5 has -10 and -35 sequences that correspond exactly to the consensus for early promoters, as does the promoter in fragment 10(4), but none of the other cloned promoters. Some of the other fragments contain the -10 or -35 consensus, but not the other. Fragment 10(3) has both of these consensus sequences but not in the correct relationship to each other. Fragments 10(2), 10(8), 10(13) and 10(24) are identical. The same is true for the two promoter fragments from EcoRI*18, 18(3) and 18(13). The sequences of these cloned fragments are shown in Table 3, except for Pe4 and Pe5, which are included in Figure 8.

**Cloning and Sequencing of SPO1 Early Genes e3 and e22.**

The sequences surrounding promoters Pe4, Pe5 (above) and Pe6 (located at the left end of EcoRI*10, provided by E. P. Geiduschek, were used to design appropriate primers. A 297-bp fragment between promoters Pe4 and Pe5, and an 890-bp fragment between promoters Pe5 and Pe6 were amplified by PCR and cloned into the SmaI site of pUC19. Several clones from each cloning reaction were sequenced. The sequences of these two PCR fragments and promoters Pe4 and Pe5 constitute a continuous segment of about 1.5 kb, containing all of EcoRI* fragment 26 and the right end of EcoRI* fragment 15 (Fig. 7).

The complete sequence of this 1.5 kb segment is shown in Figure 8. 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 (Mountain, 1989), and each promoter had an
<table>
<thead>
<tr>
<th>Fragment</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>10(3), 249 bp</td>
<td>5' GATCTGGATG AGAAGCTTAT GAAGTATATC AGACGGGAGG AATAAAAAATG GAGCAAGCAA CTGTAGTATC TCAGGTAAGCC ATTTGATGTAG GACCGAAGGG TATCGGTTTC TCTTTTGAGG GCATGGGATAA TGACTTAGCT GAGCTTATTA GTCAAGGCTAA GGAATTTGAG TCTATAGAA AAGGATTCCT CACCAATGAT TCTAGCACC TGGACTATTGC GTCTAAGAAG AAGATTTGCTT TTGTTGACA 3'</td>
</tr>
<tr>
<td>10(4), 442 bp</td>
<td>5' GATCTAGAGT TTGAAGTTCA TTGGATCATG TTTATGGAT CGTGGATGTG AACGAAGAGG GTCTATTCTG CCTAGGTTGG GTGTCTGATA TCTCCCTGTA ATCCCTCCA AATATGCTGG GCGGTGGGGG TGAGTTGGTC GAGTCTATAG AGGATGTCTT GTCTGAGCAT GCGGGCTCTA TTGGATACG GGTCTGAGTC TGATGGAGGG GCGGGTCTTC CCGCCCTACT TTTCGTATA TACGCTGGAC TTCTGCTATC AAGTGTCGA A TAAATTATAGT AACATCACAG AGAAATAAGAA CGGACTATCA GAGACAATAAA TAAACAAAGG AGCGGTGTA ATGCCATATT CTAAAATCAC TGTACCAATT TTATGATAGG AAGGGTAAAC AGAGTGGGAT GTTTATGATG TTATGAGGGGA AACTCATCCG CACTCGTGG AG 3'</td>
</tr>
<tr>
<td>10(2, 8, 13, 24)</td>
<td>5' GATCCACCTCC ATGTACCTCC CATTTATTTGT ATAAATAAAAAG GGGTACGCGA GCCACCCCC TTGACATTAC TTCTTACGC TTTTTCTTGC GAGGGCTCTC GCTATCGGC GTCTA 3'</td>
</tr>
<tr>
<td>18(3, 13), 39 bp</td>
<td>5' GATCACAGTG GTATTATGAT GTTGAATGATC 3'</td>
</tr>
</tbody>
</table>

a. Color indicates the relative darkness of clones containing these fragments on X-gal plates. Early promoter consensus sequences are bold-underlined, sequences that might be the -10 or -35 region of a early promoter are shown in bold-italic.
Figure 8. Complete sequence of the 1.5-kb segment. The -10 and -35 regions of promoters Pe4 and Pe5 are indicated by double underlines, the ribosome-binding sites by single underlines, and the putative upstream activation sequences (UAS) by bold italics. The deduced amino acid sequences of e3 and e22 are shown under their nucleotide sequences. The EcoRI sites are shown in bold, overlined. Nucleotide substitutions in mutations discussed in the text are shown above the wild-type sequences that they replace, and are marked by * under the wild-type sequences. In e3, acidic and basic amino acids are shown in shadow and bold-underline, respectively.
1 GATCAGTCCT TTGTAGGTC TAGGATGTA CTACATTTTC AAGACAGCTA TCTGAAATTG 60
61 AAACCCCTGTC ACTAGAGACT TGTCAATTGAG GCACAGCTAT GTGCACTGG GTACACAGCAA 120
121 AAGACTGGCT CAGTAGCCGC AATATTTCCT CCGATTGGGGG TAAAGAGAG TGTGGCCTAG 180
181 AGAGATCCAC GCTGTGAAA AATTTTGACAA AAGGATTTT GCATTCCCTA CAGGGTCTTCT 240
241 AATTAATTAA TTACAGCGGC GGGCAACCCC GCCAGTAGCTC TACAGGGTA AAGGACCGGT -35
-10
301 TAAC ATG ATC GAA TTA TCT GAA AGA CAG GAG TTA TTG CAG GTG CTT 349
1 Met Ile Gln Leu Ser Glu Arg Gln Gln Asp Leu Leu Gln Val Ala 15

350 GAG AAG TAT GAG CAG TGC CAC ATA GAA TGG TAC AGA GCG CAG TCC AGA CTA 400
16 Glu Lys Tyr Glu Gln Cys His Ile Glu Phe Tyr Thr Ala Gln Ser Arg Leu 32

401 TTC GGC ACT GAA ATT ATG GGT GAA GTG GTC AAG ACC TCA ACG ACT GTA 451
33 Phe Gly Thr Met Gly Val Leu Thr Ser Ser Thr Ala Tyr Thr Leu 49

452 AAA ATA GCG CAC CCT GAA GAA GAT TTA TTT GAG GTA GCC GCT GCC TAC GTA 502
50 Lys Ile Ala His Pro Glu Glu Asp Leu Phe Glu Val Ala Ala Tyr Leu 66

503 GCT TCC AAG AAA GAT ATC CTA ACA GCA CAA GAG GCG AAA GAT GGT TTG TTC 553
67 Ala Ser Lys Asp Ile Leu Thr Ala Glu Arg Lys Asp Val Leu Phe 83

554 TAC ATC CAG AAC ATC TGC TCC TAA ATTCCT GAAAAATTTT GCCAAAAGATT 604
84 Tyr Ile Glu Asn Asn Leu Cys OCH 90

605 GGTGAGTTTA TCTAGAGGTGGGGCTAAAT ATCTTAAGCA ACAGCAGGAG GCTACTGCTG 664
-35 # -10
665 CTAGGCGTCC CCGTAGCCGC GGGCGCAGCT GGAGGGCTAG ACAGCGCTAA ATCAGGAAGAT

TAG
725 GATAAAGGAG AGATTGCAAG ATG GCT AAA TCA AAT AAC GTG TAC GTG GTA AAT 776
1 Met Ala Lys Ser Asn Asn Val Tyr Val Val Asn 11

777 GGT GAA GAA AAG GTT TCA ACT GTT GCA GAG GGT GTT GCT AAG GCT GTA 827
12 Gly Glu Glu Lys Val Ser Thr Leu Ala Glu Val Ala Lys Val Leu Gly Val 28

828 AGT GTG GTA TCT AAG AAG GCT GAG GAA GCG AAT TAG GTT GTA GCT GCT 878
29 Ser Arg Val ser Lys Lys Asp val Glu Glu Gly Lys Tyr Asp Val Val Val 45

879 GAG GAA GCC GCT GTT TCT CTA GCT GAT ACT GAG GAA GGT GTG GAA GCT ACG 929
46 Glu Glu Ala Ala Val Ser Leu Ala Glu Thr Glu Glu Val Val Glu Val 62

920 GTA ACA GAG GAA GAG GAC ATG CTA GAA GGC GTG GAA GTT GTC GAG GAT GAC 980
63 Val Thr Glu Glu Glu Asp Ile Leu Glu Gly Val Val Glu Val Glu Asp Glu 79

981 GAA GAG GAA GGC GCT GAA GAT GTA GAG GAG CCT ACA TCT GAA GAG GAT 1031
80 Glu Glu Glu Glu Ala Ala Glu Asp Val Glu Glu Pro Thr Ser Glu Glu Asp 96
1032 TCT GAG GAC GAA TGG GAA GAA GCC TAC CCA GTT GCT ACT GAA GTT GAG GAA 1082
  97 Ser Glu Asp Glu Trp Glu Glu Gly Tyr Pro Val Ala Thr Glu Val Glu Glu 113
1083 GAT GAG GAC GAA GAG ATT GAG TAC CCT GAA GTC GCT GAG TTC GAG GAC GAA 1133
  114 Asp Glu Asp Glu Glu Ile Glu Tyr Pro Glu Val Gly Asp Phe Glu Asp Glu 130
1134 AAG GCT ATC AAG AAC TAC ATC AAA GGG CTG ACA GAC GAA GAG CTT CAA GCA 1184
  131 Lys Ala Ile Lys Lys Tyr Ile Lys Gly Leu Thr Asp Glu Gln Leu Gln Ala 147
1185 TGG TCT GAG TTG GAA GCC GCT GAG TGG GTG GAA AAC GAA CAC GCG AAG ATC 1235
  148 Trp Cys Glu Leu Glu Gly Ala Glu Trp Val Glu Asn Glu His Arg Asn Ile 164
1236 AAC GGT ATG AGA ATG GCT ATG CCT ATC AAG GCT GTA CAC TTC CCT GAG TTG 1286
  165 Asn Arg Met Arg Met Ala Met Ala Ile Lys Ala Val His Phe Pro Glu Leu 181
1287 GCT AAA AAG CTT AGC AAA AAG AAC TCT AAG TAC GCT GAG TAC ACT AGA 1337
  182 Ala Lys Lys Pro Ser Ser Lys Lys Lys Ser Lys Tyr Ala Glu Tyr Thr Thr 198
1338 GAA GAA CTA GTT GAA ATG GCT ATC GAC AAT AAC GTT GAG GTA CGT GAC GAC 1388
  199 Glu Glu Leu Val Glu Met Ala Ile Asp Asn Val Glu Val Arg Asp Asp 215
1389 AAG GGC AAT GAG CGC ATC GTT AGA AGT TAT ACC ATA ATC GCA TTA CGT GAA 1439
  216 Lys Gly Asn Glu Arg Ile Leu Arg Met Tyr Thr Ile Ile Ala Leu Arg Glu 232
1440 GCG GGG CTA ATC ACT TAA GGGAGCCTCA AGGCTGGCCT TAGTTAATTC TAATTGCGTT 1497
  233 Ala Gly Leu Ile Ser OCH 237
1498 AGTACAGAGAATTGGCTA CTTGTCCGA TACA 1531
apparent upstream activation sequence (Frisby and Zuber, 1991).

30% of the amino acids in the e3 protein were acidic, including 55 glutamate and 16 aspartate. 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.

A spontaneously derived clonable mutant of EcoRI* fragment 26 (Lee et al., 1980a) in plasmid pCL2 was also sequenced. It has a C to T substitution at the -10 region of promoter Pe5, and a nonsense mutation at codon 71 of gene e3, changing a Glu (GAA) to a stop codon (TAA). Since the -10 mutation did not decrease promoter activity (Lee et al., 1980b), it was presumably the nonsense mutation that permitted cloning. This mutant e3 gene was named e3m71.

CONCLUSIONS AND DISCUSSION

The nucleotide sequence of a 1.5 kb segment of the SPO1 terminal redundancy, including promoters Pe4 and Pe5 and genes e3 and e22, was determined. Early promoters Pe4 and Pe5, just upstream of genes e22 and e3, respectively, are among the strongest promoters known (Talkington and Pero, 1977; Lee et al., 1980a, b; Romeo et al., 1981). Each has, just upstream of its -35 region, two strings of five adenines, six base pairs apart, the sort of structure that has been termed an upstream activator sequence (UAS) (see Table 4) because it is essential for maximum activity
of certain promoters, possibly because of curvature that it imparts to the DNA (McAllister and Achberger, 1988, 1989; Frisby and Zuber, 1991). Indeed, deletion of this UAS from Pe5 reduced its activity by about 80% (our unpublished results). None of the other early promoters sequenced, including the very active 10(4), have such an upstream sequence. The Alu 156 promoter, one of the primary promoters used for the study of UAS function (McAllister and Achberger, 1988, 1989), is from the closely related phage SP82. This promoter is nearly identical to promoter Pe5, indicating that the two are possibly descended from the same promoter in a common ancestor.

Both e3 and e22 also have strong ribosome-binding sites, with ΔG values of -19.0 kcal/mole for e3 and -18.0 kcal/mole for e22. Unlike E. coli mRNAs, which have a relatively short Shine-Dalgarno (SD) sequence (a consensus of AGGA), a spacing of 6-12 bases to the initiation codon, and an average free energy of interaction between the SD sequence and the 3' end of the 16S rRNA of -11 kcal/mole (Gold et al., 1981), B. subtilis mRNAs usually have a more extensive complementarity between the SD sequence (a consensus of AAAGGAGGTGAT) and the 16S rRNA, with an average ΔG of -17 to -18 kcal/mole (McLaughlin et al., 1981).

The e22 gene specifies a 90 amino-acid protein, which shows no significant similarity to any known protein. We have no direct evidence that the e22 protein actually is translated, but its precise placement just downstream of an efficient promoter and ribosome-binding site and its close but not overlapping relationship with Pe5 suggest that it is. 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
Table 4. Promoter Comparison of the Upstream Activation Sequences

<table>
<thead>
<tr>
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<th>Sequence</th>
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<tbody>
<tr>
<td>Alu156</td>
<td>AAAATTCCTGAAAAAAATTTCGAAAAAAGTGTGTGACTTTCTCTACGAGGTGTGGCATATAAT</td>
</tr>
<tr>
<td>Pe5</td>
<td>AAAATTCCTGAAAAAAATTTCGAAAAAAGTGTGTGACTTTATCTACAAGGTGTGGCATATAAT</td>
</tr>
<tr>
<td>Pe4</td>
<td>ACCTGTGTGAAAAATTTCATACAAAAAGTTATGTGACTTTCCCTACAGGGTGTAATAAT</td>
</tr>
</tbody>
</table>

*a.* Runs of adenines are underlined, and promoter consensus sequences are bold.
significant similarities between e3 and other known genes 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) (Earnshaw, 1987); Ca++-binding (Leberer et al., 1989); and/or interaction with other proteins; but for many the activities are unknown. 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 (Altschul et al., 1990; Gribskov and Devereux, 1991)] included: mouse nucleolar transcription factor 1 (Hisatake et al., 1991); varicella-zoster virus alpha trans-inducing factor (Davison and Scott, 1986); the human or mouse major centromere autoantigen B (Sullivan and Glass, 1991); pseudorabies virus gene RSp40 (Zhang and Leader, 1990); Drosophila troponin T (Fryberg et al., 1990); the erythrocyte surface antigen receptor (Favaloro et al., 1986) and the glutamic-acid-rich protein (Triglia et al., 1988) of Plasmodium falciparum; the human sarcoplasmic reticulum histidine-rich calcium binding protein (Hofmann et al., 1991); the rabbit ryanodine receptor (Takeshima et al., 1989); and bovine prothymosin α (Panneerselvam et al., 1988). Such diversity clearly precludes drawing any conclusions about e3 function on the basis of its similarity to other proteins.
Chapter 4. Effects of e3 on Cell Growth and Macromolecular Synthesis.

INTRODUCTION

Because intact EcoRI* fragment 26 was not clonable, I hypothesized that the e3 product is involved in the shutoff of host biosynthesis and is therefore toxic to host cells. To test this hypothesis, I cloned the e3 gene under control of an inducible promoter, and analyzed the effect of e3 expression on host functions.

RESULTS

Cytotoxicity of e3 Gene Product. The e3 gene could be cloned under repressed conditions, in the E. coli/B. subtilis expression vector pPW19 (Fig. 5). Expression of the cloned e3 gene, by induction with IPTG, prevented colony formation in either E. coli or B. subtilis (Table 5). 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 generation and characterization of this mutant are described in Materials and Methods and in Chapter 6.) The e3m71 gene, which has a nonsense mutation at codon 71, caused a small reduction in colony-forming efficiency when expressed from the same promoter, 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.

Inhibition of Host DNA, RNA and Protein Synthesis by e3. Figures 9 and 10 show the effects on E. coli DH5, when expression of e3...
Table 5. Effect of e3 Expression on Colony Formation.\textsuperscript{b}

<table>
<thead>
<tr>
<th>Plasmid present\textsuperscript{a}</th>
<th>Bacterial Strains</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{E. coli} DH5</td>
<td>\textit{B. subtilis} CB-10</td>
</tr>
<tr>
<td>pPW19</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td>pPW19-e3</td>
<td>9.6 x 10\textsuperscript{-6}</td>
<td>2.9 x 10\textsuperscript{-3}</td>
</tr>
<tr>
<td>pPW19-e3\textsuperscript{m3}</td>
<td>1.09</td>
<td>0.85</td>
</tr>
<tr>
<td>pPW19-e3\textsuperscript{m71}</td>
<td>0.12</td>
<td>0.47</td>
</tr>
<tr>
<td>pPW19-lacZ</td>
<td>0.98</td>
<td>1.24</td>
</tr>
</tbody>
</table>

\textsuperscript{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. The symbol “e3” represents the wild-type e3 gene; “e3\textsuperscript{m3}” and “e3\textsuperscript{m71}” are mutant e3 genes, with nonsense mutations at codons 3 and 71, respectively; “lacZ” is the \textit{E. coli} lacZ gene in translational fusion with a \textit{B. subtilis} ribosome-binding site.

\textsuperscript{b} Each plasmid was introduced into both \textit{E. coli} DH5 and \textit{B. subtilis} CB-10, and the strains produced were plated with or without IPTG. The numbers shown are the ratios of the number of colonies formed in the presence of IPTG to the number of colonies formed without IPTG.
Figure 9. Effect of e3 expression on *E. coli* Growth. DH5 cells containing various pPW19-derivative plasmids were grown at 37°C in NY medium with 10 μg/ml of chloramphenicol to early exponential phase (Klett 35-50). 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.
Figure 10. Effect of e3 expression on DNA, RNA and protein synthesis 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 the cultures, and, at various times thereafter, aliquots were pulse-labeled and counted for total [3H] incorporation as described in Materials and Methods. Open squares: no IPTG added. Filled squares: 0.2 mM IPTG added at time 0. The significance of the details of the shapes of these curves has not been established. For instance, the decrease from the two hour to the three hour points, which is seen in the open square curves in the right hand column, was not seen in all repetitions of these experiments, presumably because of variations in experimental conditions.
was induced during exponential growth. Cell growth virtually ceased, and the synthesis of DNA, RNA, and protein was gradually shut off. Figure 11 shows a similar effect on *B. subtilis* CB-10, although 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*, I do not wish to attribute any significance to the quantitative differences between *E. coli* and *B. subtilis* seen in these figures.]

**DISCUSSION**

As predicted from the unclonability of EcoRI* fragment 26, the *e3* gene product, when expressed from a multi-copy plasmid in either *B. subtilis* or *E. coli*, does cause the inhibition of DNA, RNA, and protein synthesis, and prevents colony formation. It is not surprising that a gene from a *B. subtilis* phage can shut off *E. coli* functions, since many functions in the two bacteria are accomplished by homologous molecules utilizing similar mechanisms (Sonenshein et al., 1993).

The quantitative differences between the effects of *e3* on *E. coli* and *B. subtilis* can be explained by differences in the behavior of the plasmid in the two bacteria. First, the uninduced Spac-I promoter in pPW19-e3 is not as completely repressed in *E. coli* DH5 as in *B. subtilis* CB-10, as indicated by northern blots measuring the amount of *e3* transcription without induction (data not shown). This activity of the Spac-I promoter results in
Figure 11. Effect of e3 expression on DNA, RNA and protein synthesis 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 the cultures, and, at various times thereafter, aliquots were pulse-labeled and counted for total [3H] incorporation as described in Materials and Methods. Open squares: no IPTG added. Filled squares: 0.2 mM IPTG added at time 0. The significance of the details of the shapes of these curves has not been established. For instance, the decrease from the two hour to the two and half hour points, which is seen in the open square curves in the right hand column, was not seen in all repetitions of these experiments, presumably because of variations in experimental conditions.
slower growth for DH5(pPW19-e3) than for controls, even without IPTG, as seen in Figures 9 and 10. It was also noticed that the colony size of DH5(pPW19-e3) was less than half the size of DH5(pPW19-lacZ) after the same time of incubation on plates without IPTG.

Secondly, pPW19-based plasmids are structurally less stable in B. subtilis than in E. coli, resulting, for instance, in the higher frequency of IPTG-resistant CB-10(pPW19-e3) colonies than DH5(pPW19-e3) colonies (Table 5). This instability possibly contributes to the less efficient shutoff of macromolecular synthesis by e3 in CB-10 than in DH5 (compare Figures 10 and 11). B. subtilis plasmids of this type are known to be particularly labile to excision of the DNA between short direct repeats (Bron et al., 1991; Janniere et al., 1990), several of which occur in the Spac-I promoter and the e3 gene. Of plasmids isolated from IPTG-resistant CB-10(pPW19-e3) clones, most had deletions in the Spac-I promoter and/or e3 gene (data not shown). The same was true for plasmids from IPTG-resistant DH5(pPW19-e3) clones, although the deletion occurred at a much lower frequency.

The fact that shutoff can be caused by e3 expression in uninfected cells does not show that e3 is responsible for the shutoff that occurs during SPO1 infection. Proteins such as the products of T4 gene 32 and 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 (Krisch and Selzer, 1981; Maiti et al., 1991), and this could be true for e3 as well. Arguments against this possibility are presented in the next several chapters.
Chapter 5. Identification of the Cellular Target of the e3 Product by Characterization of E. coli Genes that Provide Resistance to e3.

INTRODUCTION

In Figures 10 and 11, the shutoff of RNA synthesis took place somewhat more rapidly than that of DNA or protein synthesis. Thus, it is possible that RNA synthesis is the primary target of the e3 product, and that the effects on DNA and protein syntheses are secondary. However, all of the shutoffs took place so slowly that no definitive conclusion could be made. To obtain more specific insights into the target of e3 action, E. coli mutants resistant to the effects of e3 were isolated and studied. Normally, expression of the plasmid-borne e3 gene kills either B. subtilis or E. coli (Table 5). A few IPTG-resistant colonies can form, most of which have altered plasmids, which are presumably unable to express normal amounts of the e3 product. However, e3-resistant mutations can also occur on the bacterial chromosome, at such a frequency that they can be detected among all the IPTG-resistant E. coli clones, but not among the much more frequent IPTG-resistant B. subtilis clones. Characterizing these chromosomal mutant strains should identify mutations that restore cell growth, and thus identify the cellular target(s) of the e3 gene product.

An e3-resistant E. coli DH5 strain was isolated, and plasmid libraries of its genomic DNA were screened for capacity to protect against e3-induced cell killing. At least two genes were found to provide such protection. Each restored cell survival in the presence of e3 expression.
RESULTS

Screening and Identification of an e3-Resistant E. coli DH5 Strain. Because of the greater instability of pPW19-e3 in B. subtilis, and because the effect of e3 was at least as great in E. coli as in B. subtilis (discussed in Chapter 4), E. coli DH5 was used in the selection of e3-resistant strains.

As described in the Materials and Methods, about $3.7 \times 10^7$ DH5(pPW19-e3) cells were screened on plates containing IPTG and Cm, and a few hundred resistant colonies were obtained. Of the 27 clones that were further characterized, 24 had suffered alterations of the plasmid, as shown either by a change in restriction fragments produced or by the ability to form colonies on IPTG plates. The three whose plasmids were apparently unchanged were cured of plasmid by growth in the absence of selection, and tested for e3-resistance by transformation with wild-type pPW19-e3. One of the three showed such resistance and was named DH5R. Typical data from this screening process are shown in Table 6.

As shown in Figure 12, the IPTG-induced killing that takes place in DH5(pPW19-e3) did not take place in DH5R(pPW19-e3). Even in the absence of IPTG, DH5R(pPW19-e3) grew faster than DH5(pPW19-e3), again reflecting the incomplete repression of e3 transcription in wild-type E. coli cells.

Construction of Plasmid Libraries from DH5 and DH5R Genomic DNAs. As described in the Materials and Methods, DH5 and DH5R chromosomal DNAs were isolated and partially digested with restriction enzyme HpaII. In each case, two partial digests were produced, with size distributions about 2-4 kb and 4-10 kb. Each of these digests, was
Table 6. Screening and Identification of an e3-Resistant DH5 Strain

A. Transformation of plasmids, isolated from IPTG-resistant clones, into competent DH5 cells, using pPW19-e3 and H₂O as controls:

<table>
<thead>
<tr>
<th>Plasmid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>-IPTG</th>
<th>+IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>~200</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>~100</td>
<td>~100</td>
</tr>
<tr>
<td>C</td>
<td>~500</td>
<td>~500</td>
</tr>
<tr>
<td>pPW19-e3</td>
<td>~1000</td>
<td>0</td>
</tr>
<tr>
<td>H₂O</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

B. Transformation of plasmid pPW19-e3 into competent DH5<sup>R</sup>, using DH5 competent cells as control:

<table>
<thead>
<tr>
<th>Competent Cells</th>
<th>-IPTG</th>
<th>+IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5</td>
<td>~500</td>
<td>0</td>
</tr>
<tr>
<td>DH5&lt;sup&gt;R&lt;/sup&gt;</td>
<td>225</td>
<td>155</td>
</tr>
<tr>
<td>DH5&lt;sup&gt;R&lt;/sup&gt;(repeat)</td>
<td>220</td>
<td>165</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are shown for three of the 27 plasmids tested. A is the plasmid from the strain ultimately designated DH5<sup>R</sup>. B and C are from two of the other strains, with altered plasmids.

<sup>b</sup> "~" represents estimated numbers.
Figure 12. Protection against e3-induced killing by the DH5R mutation. Exponentially growing cultures of DH5(pPW19-e3) (squares) and DH5R(pPW19-e3) (circles) were split into two cultures. At time 0, 0.2 mM IPTG was added to one (filled symbols) of the cultures, and, at various times thereafter, aliquots were diluted and plated on plates containing 10 μg/ml of chloramphenicol, but no IPTG. These plates were incubated at 37°C for one to two days. Numbers of colony forming units in the undiluted culture are shown as a function of the time after addition of IPTG. The significance of the differences in detail between the three upper curves has not been established.
then cloned into the ClaI site of plasmid pACYC177 (Fig. 6), forming library DNA DH5Lib-1 and DH5\textsuperscript{R}Lib-1 (the 2-4 kb inserts from DH5 and DH5\textsuperscript{R} genomic DNA, respectively), and DH5Lib-2 and DH5\textsuperscript{R}Lib-2 (the 4-10 kb inserts). The best results were obtained by ligating DNAs at a total concentration of 30 \(\mu\text{g/ml}\) with a vector/insert ratio of 2:1. Maximum transformation efficiency (10\(^9\)-10\(^10\)/\(\mu\text{g DNA}\)) was achieved by electroporation.

**Testing and Screening of Plasmid Libraries.** The quality of the libraries was first tested by determining the frequency with which clones were obtained which expressed either of two *E. coli* genes, \(\text{lacZ}\) (about 3 kb) (Kalnins et al., 1983) and \(\text{cysB}\) (about 1 kb) (Ostrowski et al., 1987). DNAs from each library were transformed into competent MC4100, which lacks the lactose operon, and the frequency of \(\beta\)-galactosidase producing clones was measured as the frequency of blue colonies on X-gal plates. Except for library DNA DH5Lib-1, which gave no blue colony in a total of 3 x 10\(^4\) transformants, all the library DNAs had the \(\text{lacZ}\) gene cloned at a frequency of 1-10 x 10\(^{-4}\). DH5Lib-1 and DH5\textsuperscript{R}Lib-1 were also transformed into MDA4762, an *E. coli* cysteine and tryptophan auxotroph, and selected for colonies that grew on plates without cysteine. Both of these library DNAs had the \(\text{cysB}\) gene cloned at a frequency of 3-4 x 10\(^{-4}\). Thus, any clonable *E. coli* sequence should appear at a frequency greater than 1 in 10,000 among clones made from these libraries.

DH5\textsuperscript{R} library DNAs were transformed into competent DH5(pPW19-e3) cells and IPTG-resistant clones were selected on plates containing IPTG, Ap and Cm. DH5 library DNAs were used as controls. Twenty-
five IPTG-resistant colonies were obtained and analyzed. For each, the
two plasmids present were isolated separately, as described in Materials and
Methods. The CmR plasmid, carrying the e3 gene, was tested by
transformation of DH5, to determine whether it retained IPTG-inducible
killing activity. The ApR plasmid, carrying a cloned fragment from the
DH5R or DH5 genome, was tested by transformation of DH5(pPW19-e3) to
determine whether it protected against the IPTG-induced killing activity.
Table 7 summarizes the results. Seven clones retained active CmR plasmid
pPW19-e3, and four of these had ApR plasmids which conferred IPTG-
resistance upon DH5(pPW19-e3). These four represent cloned genes which
protect against the killing effect of e3. Three of these, from DH5R library
DNAs, were named pPW40, pPW50 and pPW60 (pPW60 was from
DH5RLib-1), and one, from DH5Lib-1 DNA, was named pPW70. The
sizes of cloned fragments in these plasmids were approximately 8.8, 7.3,
2.0 and 4.1 kb, respectively. The other three of the clones which retained
active pPW19-e3 presumably have new IPTG-resistant chromosomal
mutations, and have not been characterized further.

**Mapping of Cloned Fragments.** The ends of the cloned
fragments in plasmids pPW40, 50, 60 and 70 were sequenced, using
primers CLA-L and CLA-R (Table 2), which were based on vector
sequences adjacent to the ClaI cloning site. The sequences obtained were
used to probe the GenBank database, in each case revealing virtual identity
with a known *E. coli* sequence.

pPW40 and pPW50 contain overlapping fragments, each of which
includes the *rpoB* gene and several nearby genes around 90 min on the *E.
coli* chromosome. The sequence at the right end of each cloned fragment
Table 7. Screening and Identification of e3-Resistant clones from Constructed Libraries

<table>
<thead>
<tr>
<th>Library</th>
<th>Number of Clones Screened</th>
<th>IPTG-Resistant Clones</th>
<th>Clones with Intact pPW19-e3 Plasmid</th>
<th>Clones with e3-Resistant Gene on the Ap&lt;sup&gt;R&lt;/sup&gt;Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5Lib-1</td>
<td>110,000</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DH5&lt;sup&gt;R&lt;/sup&gt;Lib-1</td>
<td>200,000</td>
<td>11</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>DH5Lib-2</td>
<td>13,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DH5&lt;sup&gt;R&lt;/sup&gt;Lib-2</td>
<td>127,000</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
matched the sequence near the beginning of *E. coli* gene *rpoC* (Ovchinnikov et al., 1982), although the sequence in pPW50 extended about 100 bp further into *rpoC* than that in pPW40. The left end of the cloned fragment in pPW40 was mapped to the middle of *E. coli* gene *tufB* (Sekiya et al., 1976), while the left end of the pPW50 fragment corresponds to the end of gene *nusG* (Downing et al., 1990). The restriction map of each cloned fragment showed complete agreement with the map of the corresponding region of the *E. coli* genome, as shown in Figure 13.

The sequences of the ends of the cloned fragment in pPW60 were mapped to regions flanking genes *sfs1-dksA* around 3.6 min on the *E. coli* chromosome (Kawamukai et al., 1991; Kang and Craig, 1990). Again, the restriction map of the cloned fragment in pPW60 corresponded exactly to the map of the corresponding section of the *E. coli* genome, as shown in Figure 14.

The sequence of one end of the cloned fragment in pPW70 was mapped to the left end of an unannotated sequence [about 2.2 kb, containing the *ssrA* gene, which specifies a small stable RNA (Chauhan and Apirion, 1989) at the middle of this sequence] whose map position was estimated to be around 57.5 min (Rudd, 1992). The restriction map of this cloned fragment differed somewhat from that for the corresponding region of the chromosome. More than half of that region has not been sequenced, and *ssrA* is the only gene identified in that region. The sequence (88-bp) of the other end failed to show similarity to any known sequences. pPW70 was not further characterized.

Thus, the cloned fragments in pPW40, 50 and 60 were either wild-
Figure 13. Maps of the \textit{rpoB} region and cloned fragments from that region. The top line shows map distances in kb. "0" kb represents the left end of the cloned fragment in pPW50. The second line is the restriction map of this region of the \textit{E. coli} genome taken from Kohara et al. (1987). A=AvaI, B=BglII, BII=BglIII, C=Clai, H=HindIII, K=KpnI, P=PvuII, R=EcoRI, V=EcoRV. Genes and promoters are shown as boxes and arrows below the restriction map, with transcription direction indicated by the arrows (An and Friesen, 1980; Downing et al., 1990; Post et al., 1979; Ovchinnikov et al., 1981,1982). Lines at the bottom represent the lengths of cloned fragments related to the map, with enzymes used in subcloning shown at the ends, and the name of each recombinant plasmid shown at the right end.
Figure 14. Maps of the *dksA* gene region and cloned fragments from that region. The top line shows map distances in kb. "0" kb represents the first nucleotide of the cloned fragment in pPW60. The second line is the restriction map of this region of the *E. coli* genome, taken from Kohara et al. (1987). Genes and promoters are shown as boxes and arrows below the restriction map, with transcription direction indicated by the arrows (Kawamukai et al., 1991; Kang and Craig, 1990). Lines at the bottom represent the lengths of the cloned fragments related to the map, with the enzymes used in subcloning shown at the ends and the name of each recombinant plasmid shown at the right end. C=Clai, H=HincII, P=PvuII, and V=EcoRV.
type genomic fragments or fragments containing mutations small enough that they did not detectably affect the fragment size. Interestingly, all the fragments were cloned in such an orientation that genes on the fragments would be correctly transcribed by the Km promoter on the vector. For clarity, genes and promoters on these fragments were labelled X' and P'x, respectively, in order to discriminate them from their wild-type counterparts, X and P, respectively.

**Subcloning for Identification of Responsible Genes in Cloned Fragments.** The cloned fragments in plasmids pPW50 and pPW60 were subcloned in order to identify the gene or genes whose products provided protection against e3. As shown in Figure 13, the 7.3 kb fragment in pPW50 contains the complete rpl'K'A'J'L'-rpoB' gene cluster with promoters P'L11 and P'L10. The rplK, rplA, rplJ and rplL genes specify ribosomal proteins L11, L1, L10 and L7/12, respectively (Post et al., 1979), and the rpoB gene specifies the RNA polymerase β subunit (Ovchinnikov et al., 1981). Subfragments containing various subsets of these genes, as shown in Figure 13, were cloned into pACYC177, in each case inserting at a site within the Km gene, such that genes in the cloned fragments were oriented in the same direction as the Km gene, forming plasmids pPW51, pPW52 and pPW53.

Each of these pPW50-derivatives was transformed into DH5(pPW19-e3) and tested for its capacity to protect against killing by e3. As shown in Figure 15A, pPW51 provided protection but pPW52 and pPW53 did not. Since pPW51 includes only the rpoB and rplL genes, and the rplL gene is included in pPW52, we concluded that the rpoB' gene product, presumably
expressed from the Km gene promoter, is responsible for counteracting the effect of e3 protein.

The cloned fragment in pPW60 contains two small genes, *sfsA*′ and *dksA*′, and their promoters, P′₁ and P′₂ (Fig. 14). The *sfsA* and *dksA* genes specify a regulatory factor for maltose metabolism and a *dnaK* suppressor, respectively (Kawamukai et al., 1991; Kang and Craig, 1990). Four subclones of pPW60, pPW61 through pPW64, were obtained. As shown in Figure 15B, pPW62 or pPW63, each of which includes the *dksA*′ gene, provided protection, while pPW61, which includes the *sfsA*′ gene, did not. pPW64, which includes all but the first 36 codons of the *dksA* gene (which encodes a protein of 151 amino acids), in the same reading frame as the Km gene, appeared to provide a small degree of protection. Thus, the intact *dksA*′ gene, expressed from its own promoter and/or the Km gene promoter, was capable of protecting against the killing effects of e3.

**The Nature of *rpoB*′ and *dksA*′ Genes.** Since the protective genes were cloned from a mutant e3-resistant *E. coli* strain, we presume that one of the protective genes carries the mutation responsible for the e3-resistance, and that the other is a wild-type gene, which is protective only because of overexpression. To determine which was which, we obtained the wild-type *rpoB* and *dksA* genes from R. Landick (Landick and Stewart, 1990) and E. Craig (Kang and Craig, 1990), respectively. Cloned into the Km gene of pACYC177, forming pPW54 and pPW63wt, respectively, the wild-type *dksA* gene provided protection against killing by e3, but the wild-type *rpoB* gene did not. Thus, although I have not demonstrated it by sequencing, I believe that the selected *rpoB* gene has a mutation that makes
Figure 15. Protection against e3-induced killing by the \textit{rpoB} and \textit{dksA} genes. Exponentially growing cultures of DH5(pPW19-e3), also containing (A) pACYC177, pPW40, pPW50 or various subclones of pPW50; or (B) pPW60 and its subclones, were each split into two cultures. At time 0, 0.2 mM IPTG was added to one of each pair of cultures (bottom panel, labelled with “+”), and, at various times thereafter, aliquots were diluted and plated on plates containing 50 $\mu$g/ml of Ap and 10 $\mu$g/ml of Cm, but without IPTG. Plates were incubated at 37°C for 1-2 days before colonies were counted. The small differences seen between curves whose overall structures are similar may not be significant. This includes: (1) all differences between curves in the upper figure of 15A; (2) all differences between curves in the upper figure of 15B; (3) differences between curves with filled symbols in the lower figure of 15A; (4) differences between curves with open symbols in the lower figure of 15A; (5) differences between curves with filled symbols in the lower figure of 15B; and (6) the difference between curves with open symbols in the lower figure of 15B. A possible explanation of the latter difference, if it is in fact significant, is discussed in the text.
it e3-resistant. The wild-type dksA gene must depend on overexpression for its protective activity.

CONCLUSIONS AND DISCUSSION

DH5R and Its Genomic Libraries. A spontaneous e3-resistant mutant of DH5, DH5R, was derived under e3 selective pressure at a frequency of about 1.0 x 10^-6. Genomic DNAs from DH5 and DH5R, with average sizes of 2-4 kb and 4-10 kb, were formed into genomic libraries by cloning in plasmid pACYC177. pACYC177 has two important characteristics that make it appropriate for this cloning: (1) a p15A-derived replication origin that is compatible with plasmid pPW19-e3, which has a pBR322 origin; and (2) two antibiotic-resistant selective markers, Ap and Km, permitting selection independently of selection for plasmid pPW19-e3, which specifies Cm-resistance.

According to Clarke and Carbon (1976), the probability of having any given DNA sequence in a library can be calculated from the equation:

\[
\frac{\ln(1-P)}{N} = \frac{\ln(1-f)}{N}
\]

where \( P \) is the desired probability, \( f \) is the fractional proportion of the genome in a single recombinant, and \( N \) is the necessary number of recombinants. Therefore, to achieve a 99.9% probability (\( P = 0.999 \)) of having a given DNA sequence represented in a library of 4-kb fragments from E. coli genome (4.7 x 10^6 bp), \( N \) is approximately 8000. This number is close to those (less than 10,000) obtained by testing the
frequency of having \( lacZ \) gene or \( cysB \) gene cloned, indicating that the library DNAs were efficiently constructed and completely representative.

**Cellular Target of e3 Function.** It appears that either the expression of a mutant form of the *E. coli* RNA polymerase \( \beta \)-subunit, or the overexpression of a wild-type *dksA* gene, which encodes a DnaK suppressor, could protect cells from killing by the \( e3 \) gene product. These results provided the first clear evidence regarding the cellular target of \( e3 \) function.

RNA polymerase (RNAP) is an essential cellular enzyme, required not only for transcription but, indirectly, for translation and replication, and thus for virtually all cell functions. DksA is a dosage-dependent suppressor of a *dnaK* deletion mutation (Kang and Craig, 1990). Although the *dksA* protein (17.5 kD) is only one fourth the size of the DnaK protein (69 kD), its overexpression almost completely bypassed the requirement for DnaK in cell growth, suggesting that the *dksA* protein can accomplish most of the functions of the DnaK protein that are required for normal cell growth. DnaK, the only *E. coli* homolog of the eukaryotic hsp70 family and one of the best studied heat-shock proteins, functions by preventing the improper folding and aggregation of proteins, and by resolving protein aggregates caused by stress or normal growth (Georgopoulos et al., 1990; Gross et al., 1990).

The simplest interpretation is that the \( e3 \) product alters the conformation of the RNAP, decreasing its ability to transcribe host genes, that the mutation in the RNAP \( \beta \) subunit renders the RNAP resistant to distortion by \( e3 \), and that the *dksA* protein, when present in sufficient
quantity, either protects against or reverses that distortion. Supporting this interpretation is the fact that the dnaK protein has been shown explicitly to be capable of reactivating heat-denatured *E. coli* RNAP (Skowyra et al., 1990).

RNAP is a specific target for host shutoff in infection by other phages as well. As introduced in Chapter 1, T7 produces its own RNA polymerase and its gene 0.7 and 2.0 products inactivate the host RNAP. In T4 infection, the ADP-ribosylation of the RNAP $\alpha$ subunits by the *alt* and *mod* gene products, and the direct interaction of the *alc* gene product with RNAP reduce RNAP activity dramatically. The fact that *E. coli* mutations resistant to killing by the T4 alc product are also located in the *rpoB* gene (Snyder and Jorissen, 1988) suggests a particular similarity between e3 and alc function. However, there must be important differences as well. Alc changes the RNAP so it can transcribe hydroxymethylcytosine-containing DNA, but not cytosine-containing DNA (Snustad et al., 1988; Kutter et al., 1993). Although SPO1 also has an unusual base, hydroxymethyluracil (hmUra), the change in RNAP that occurs during SPO1 infection must be more subtle, since some host genes [eg. rRNA genes (Shub, 1966; Gage and Geiduschek, 1971a)] continue to be transcribed. Also, the alc protein alone appears to be sufficient to inhibit most host transcription in T4 infection (Kutter et al., 1993), while e3 protein appears to be neither necessary nor sufficient for host shutoff by SPO1 (see Chapter 6).

The precise mode of interaction between e3 and RNAP is a matter for speculation. RNAP consists of five essential subunits, $\alpha_2\beta\beta'\sigma$, in which the $\sigma$ factor confers transcription specificity by recognizing specific
promoters. Although the precise functions of the other four subunits are not clear, various studies have suggested that the $\alpha$ subunit (acidic) is necessary for reconstituting the active enzyme from separated subunits; the $\beta'$ subunit (basic) is involved in DNA binding; and the $\beta$ subunit (acidic) has the catalytic activity of the polymerase, since it contains most or all of the active sites for polymerization and also the binding sites for several transcription inhibitors (Reznikoff et al., 1987). We do not know whether e3 interacts directly with the $\beta$ subunit so as to inactivate the RNAP or affects other subunit(s) whose susceptibility could be suppressed by the mutant $\beta$ subunit. The net negative charge of the $\beta$ subunit and the acidic characteristic of the e3 protein make direct interaction less than optimal, but the separation of the acidic and basic amino acids into different regions of the e3 protein might permit this interaction.

Chapter 4 showed that expression of e3 inhibited DNA and protein, as well as RNA synthesis. This is reasonably understood, even if e3’s only direct effect is on RNA polymerase, since transcription is essential for both translation and replication. The relatively short half-life of mRNAs and the coupling of transcription and translation in prokaryotes mean that continuing transcription is required for continuing translation. RNAP is also important for replication initiation (Marians, 1992), in which it activates initiation not by providing a RNA primer but by generating an RNA-DNA hybrid close to the replication origin, which in turn permits open-box formation and the binding of replication factors (Baker and Kornberg, 1988).

Induction of e3 expression in DH5$^R$ caused a moderate decrease in growth rate (Fig. 12), suggesting that the protection provided by the rpoB
mutation was not absolute, and/or that e3 has other target(s) in addition to the RNA polymerase. However, growth did continue at a moderate rate after IPTG induction. In contrast, strains in which the mutant rpoB gene was provided on the plasmid, but which also had a wild-type chromosomal rpoB gene, showed considerably less growth after e3 induction (although they were protected against its killing effect) (Fig. 15). This presumably means that the mutant allele shows only incomplete dominance when present with the wild-type allele in a diploid strain, but we have not excluded the alternative possibility that a second mutation, in another gene, contributed to the e3-resistant phenotype of DH5R.
Chapter 6. Activities of e3 during Infection.

INTRODUCTION

We have shown that the e3 gene product, when expressed from a plasmid, causes the inhibition of cellular DNA, RNA and protein synthesis and ultimately kills the cell. As pointed out in Chapter 4, that does not prove that e3 accomplishes those functions during SPO1 infection. To explore the natural role of the e3 gene product in SPO1 infection, an effective approach would be to observe the behavior of an e3-defective mutant SPO1. Any difference from wild-type should reflect the function of the e3 gene product.

SPO1, with its high frequency of homologous recombination, is very efficient in permitting mutations, constructed in vitro, to be recombined into its genome (Stewart, 1993). For example, the SPO1 TF1 gene, mutated by site-directed mutagenesis, was cloned and transformed into B. subtilis. Homologous recombination was then permitted between the cloned TF1 DNA and the superinfecting SPO1 DNA. Mutant progeny phage were obtained with a frequency of 5 x 10^-4, as identified by plaque-filter hybridization (Sayre and Geiduschek, 1988).

We applied the same procedure to generate e3 nonsense mutants, although an additional step was required because of the location of the e3 gene in the terminal redundancy. The initial recombination event introduces the e3 mutation in place of one of the two wild-type alleles. Such heterozygotes segregate into wild-type or mutant homozygotes in the normal course of replication and resolution of concatemers (Cregg and Stewart, 1978), so pure mutants are produced by a second round of infection.
Here I describe the isolation of the nonsense mutant $e3^{m3}$, and the initially surprising observation that this mutation did not cause a deficiency either in host shutoff or progeny phage production. I then discuss experiments suggesting that, under some circumstances, the activity of the $e3$ product actually can be inhibitory to maximal phage production, and I describe the regulation of expression of the $e3$ gene, which may be designed to minimize this inhibitory effect.

RESULTS

Generation of an $e3$-Null Mutant SPO1--SPO1$e3^{m3}$. A new mutant of $e3$ was generated by site-directed mutagenesis, in which the third codon of $e3$ (Lys, AAA) was mutated to a stop codon (TAG) (Fig. 8). This mutant $e3$ gene was named $e3^{m3}$. To introduce the mutation into SPO1, in place of the wild-type $e3$ gene, homologous recombination was carried out by superinfecting CB-313 cells, containing the cloned $e3^{m3}$ gene, with wild-type SPO1. Progeny phage were tested for incorporation of the mutant allele by plaque-lift hybridization as described in Materials and Methods.

Of about 12,000 plaques tested, 98 hybridized to the mutant probe, implying an integration frequency of about 0.4% (considering that each genome has two target sites for integration), which was about 8 times as great as that observed by Sayre and Geiduschek (1988). The lengths of the regions of homology between the plasmid and the SPO1 genome were 108 and 760 base pairs 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 integrated into only one of the redundant regions. To permit segregation of homozygous mutants, some of these plaques were
propagated through another round of infection of CB-313, and progeny in which the e3<sup>m3</sup> mutation had replaced the wild-type allele in both copies of the TR were identified by the same filter hybridization procedures.

Figure 16 shows that the e3 mutation in fact prevented the synthesis of the e3 protein.

**The e3 Protein is Dispensable.** The nonsense mutation e3<sup>m3</sup> caused no deficiency in production of progeny phage. Figure 17 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 wild-type e3 gene product was supplied to mutant-infected cells from a plasmid, and the burst-size was smallest when wild-type e3 product was supplied by both the plasmid and the infecting phage (Fig. 18). Thus, the amount of e3 product made at 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 19 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 <sup>3</sup>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 only about 50%. Thus, it remains possible that the e3<sup>m3</sup> mutation affected
Figure 16. 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 two minutes, 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$m^3$: 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 SPO1e3$m^3$ (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 protein band (shown by the arrow) was identified both by the position of the induced band in lanes 13-15 and by the band, at the same position, that is absent in infection by the e3 nonsense mutant.
Figure 17. Effect of the e3m3 mutation on progeny production. Exponential phase cultures of CB-10, a non-suppressing strain of *B. subtilis*, were infected with SPO1 wild-type (open squares) or e3m3 (closed squares) at multiplicities of 0.01 (panel A) or 5 (panel B). Ten minutes later, the cultures were diluted 1:2 into VY containing anti-SPO1 antibody. After shaking for 5 more minutes, the cultures were diluted 1:10^5 (for low MOI) or 1:10^7 (for 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.
B

30°C

PFU

0 20 40 60 80 100 120 140

37°C

PFU

0 10 20 30 40 50 60 70 80

43°C

PFU

0 10 20 30 40 50 60 70

Time (min)
Figure 18. 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 SPO1 wild type (open symbols) or mutant e3m3 (closed symbols), and single-step growth experiments were performed as described in the legend to Figure 17. Cultures in which expression of the plasmid-borne e3 gene was induced by addition of IPTG at 10 minutes before infection are represented by circles. Cultures without IPTG are represented by squares. The small difference between uninduced and induced cultures of CB-10(pPW19-lacZ), infected with e3m3, is not significant.
Figure 19. Effect of the e3 mutation on macromolecular synthesis during infection. CB-10 cells, infected at MOI about 5.0 with wild-type SPO1 (open bars) or SPO1 e3m3 (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 minutes after infection, earlier time points are not reported in that curve. Significant counts from 5-3H uridine were incorporated into DNA late in infection (presumably having been converted to cytosine). This does 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-minute data in the bottom-left panel include a significant amount of DNA, and thus represent an overestimate of RNA synthesis at that point.
the shutoff of some subset of host RNA and/or protein syntheses, and that effect was obscured by the concomitant increase in phage-specific synthesis. However, Figure 16 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.

**Regulation of Expression of e3 and e22.** In view of the apparently inhibitory effect of excess quantities of e3 product on phage reproduction, it would not be surprising if SPO1 had found it necessary to regulate the extent of e3 expression. In fact, as seen in Figure 16, synthesis of the e3 protein began very early in infection, reached a brief peak, and then was shut off. Figure 20 shows a similar pattern for e3 RNA synthesis.

In wild-type infection, e22 behaves similarly to e3. The northern blots in Figure 21 show that both e22 and e3 were turned on early and turned off a few minutes later, and that both RNAs were degraded rapidly. Interestingly, infection with the e3 nonsense mutant resulted in the persistence of e3 RNA until much later in infection, suggesting either autogenous regulation of e3 transcription, or a direct or indirect effect of the nonsense mutation on the stability of e3 RNA. The much smaller effect in the same direction on e22 RNA may or may not be significant.

**CONCLUSIONS AND DISCUSSION**

**Apparent Redundancy of the Host Shutoff Function of e3.** The e3 nonsense mutation had no measurable effect on the shutoff of host biosynthesis during infection (Fig. 19). Moreover, although expression of a plasmid-borne e3 gene caused the inhibition of DNA, RNA and protein synthesis in uninfected cells (Chapter 4), that inhibition occurred much more
Figure 20. Temporal pattern of e3 RNA synthesis. CB-10 cells, infected with wild-type SPO1, were pulse-labeled with $[^3H]$ uridine for 2 min (represented by the horizontal bars) at various times during infection. The amount of newly synthesized e3 RNA was determined as described in Materials and Methods. The results plotted represent the average of two independent experiments, and the variations are shown by the vertical bars.
Figure 21. Northern blots of e3 and e22 RNA. CB-10 cells were infected with wild-type SPO1 or the e3 nonsense mutant SPO1e3m3 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. (A): Bottom, the gel was photographed after ethidium bromide staining and before transfer, to show that the amounts of RNA in all lanes were approximately equal. Top, the gel was transferred to a nylon filter and probed with the 32P-labeled 890 bp PCR fragment containing the e3 gene (Fig. 7). (B): the same filter was washed and rehybridized with the 32P-labeled 297 bp PCR fragment containing the e22 gene (Fig. 7).
A

SPO1wt  SPO1e3^m3

0' 3' 6' 10' 15' 25'
3' 6' 10' 15' 25'

mw

9.49
7.46
4.40
2.37
1.35
0.24

23S/16S rRNA
B

SPO1wt

0' 3' 6' 10' 15' 25'

SPO1e3

m3

3' 6' 10' 15' 25'

mw

9.49
7.46
4.40
2.37
1.35
0.24
slowly than the comparable inhibition that takes place during infection (compare Figs. 10 and 11 with Fig. 19), despite the fact that the rates of e3 protein synthesis under the two conditions were roughly comparable (Fig. 16). 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. Specifically, if, as we now believe, the e3 protein interacts with the host RNA polymerase to inhibit host mRNA synthesis, SPO1 must provide other gene products which are able to accomplish the same thing in the absence of the e3 protein.

We think it likely that a substantial number of gene products are, in fact, involved in host shutoff, both for the reasons cited in Chapter 1 with regard to the SPO1 early genes, and because that would be consistent with patterns seen for other virulent phages. T7 has at least six, and T4 at least ten, genes whose products have been shown specifically to inactivate or destroy particular host functions and/or structures (Geiduschek and Kassavetis, 1988; Kutter et al., 1993; Liu and Richardson, 1993), and each has many other genes whose expression is lethal to the host, but whose mechanisms of action are unknown (Geiduschek and Kassavetis, 1988; Studier, 1991; Kutter et al., 1993). Some of these genes have apparently redundant functions. For example, most of the ten 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 (Goff and Setzer, 1980; Geiduschek and Kassavetis, 1988; Kutter et al., 1993).

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 (Dunn and Studier, 1983; Studier, 1991; Liu and Richardson, 1993; Kutter et al., 1993). 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 (Okubo et al., 1972; Stewart, 1993).

Inhibitory Effect of e3 on SPO1 Multiplication. The e3 mutation did not diminish phage burst size, and, in fact, increased it at high MOI. Thus, the amount of e3 product made at high MOI is apparently enough to be inhibitory to the phage as well as to the host. This is plausible in view of our understanding that the e3 protein acts by distorting the host RNA polymerase so it is no longer able to synthesize host mRNA. This distortion cannot simply cause the displacement of σ^A, or render the polymerase unable to transcribe thymine-containing DNA, because host rRNA genes continue to be transcribed from σ^A-specific promoters. Thus, the distortion
must be sufficiently subtle that it permits the continued transcription both of host rRNA genes and of SPO1 middle and late genes. It would not be surprising if the e3-induced distortion were not absolutely specific in its effect, and caused a small diminution in the capacity to transcribe the SPO1 genes, resulting in decreased burst size.

Whatever the mechanism, the activity of the e3 gene product 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.

**Shutoff of e3 and e22 Transcription.** Since too much e3 product is clearly inhibitory to SPO1, it would not be surprising if SPO1 had evolved mechanisms for regulating e3 activity. In fact, transcription of both e3 and e22 is shut off after a brief period of activity. This is true for SPO1 early genes in general (Fujita et al., 1971; Gage and Geiduschek, 1971a; Talkington and Pero, 1977), and a further reason may be the same as that for the shutoff of host genes: to keep them from competing with the phage-productive middle and late genes.

Infection with the e3\(^m3\) nonsense mutant resulted in increased levels of e3 RNA that persisted until late in infection, and there was a much smaller effect in the same direction on e22 RNA (Fig. 21). It is possible that the e3 product, itself, plays a role in the shutoff of early gene activity, which was reflected in either or both of these effects. Alternatively, the effect on e22 may have been insignificant, and the effect on e3 may be due to increased stability of the e3 RNA, as a direct or indirect consequence of the nonsense mutation.
A role for e3 in early gene shutoff could be explained by its already-postulated inhibitory effect on RNA polymerase. The same distortion that inhibits host mRNA synthesis could inhibit SPO1 early gene transcription, too. There are various constraints on that proposition, and I present it only as speculation. Shutoff of SPO1 early transcription begins a short while after shutoff of host transcription, so there would have to be a difference in the effectiveness with which the two categories of transcription are shut off, perhaps based on the presence or absence of hmUra in the template. The other factor(s) which are redundant to e3 with respect to shutoff of host transcription, and those responsible for shutoff of e22 transcription even in e3m3 infection, must not be active for shutoff of e3 transcription.

Sizes of e3 and e22 Transcripts. The three most intense bands of e3 RNA were calculated to be approximately 1.4, 1.2 and 1.0 kb. E22 also has at least three major RNA species, with approximate molecular weights of 1.5, 0.9 and 0.4 kb. Careful examination of the Northern blots in Figure 21 suggests that, for the most part, the RNAs that hybridize to the e22 probe do not hybridize to the e3 probe. Thus, transcripts that begin at Pe4, or at Pe2 or Pe3 upstream of Pe4, 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 between e22 and e3 (Brennan et al., 1981), 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 (Brennan and Geiduschek, 1983).

It is known that SPO1 early transcripts are actively processed by a host enzyme having an activity similar to that of E. coli RNase III. This B.
*subtilis* RNase III recognizes stem-loop structures and cleaves at a specific adenine on the loop (Downard and Whiteley, 1981; Panganiban and Whiteley, 1983a,b). It is possible that the stem-loop structures in the *e22-e3* inter-gene region provide sites for such cleavage, which would yield *e22* RNAs of about 1.4, 0.8 and 0.4 kb from transcripts starting from promoters Pe2, Pe3 and Pe4, respectively, sizes close to those calculated from the Northern blot. However, these putative stem-loops differ substantially in length and sequence from the three well-characterized ones.

The molecular weight of the *e3* protein, calculated from its amino acid sequence is about 27 kD. The *e3* band on SDS-PAGE migrates at a position expected for 37 kD, presumably because of the high content of charged amino acids (Burton et al., 1981; Kaufmann et al., 1984; See and Jackowski, 1990).
SUMMARY

This thesis has focused on three major aspects of the characterization of bacteriophage SPO1 early genes for the understanding of host-shutoff mechanisms: (1) cloning and expression analysis of SPO1 early genes; (2) analyzing the host-shutoff ability and cellular targets of early gene products; (3) studying the roles of early genes during SPO1 infection.

The nucleotide sequence of a 1.5-kb segment in the SPO1 terminal redundancy has been determined, revealing two early genes, e22 and e3 (Fig. 7 and 8). Promoters Pe4 and Pe5, which are immediately upstream of genes e22 and e3, respectively, are among the strongest promoters known (Talkington and Pero, 1977; Lee et al., 1980a, b; Romeo et al., 1981), and each possesses a putative upstream activator sequence (UAS) (McAllister and Achberger, 1988, 1989; Frisby and Zuber, 1991). Gene e22 encodes a 90-amino acid protein, which shows no similarity to any known proteins, while e3, a highly acidic protein of 237 residues, shows significant similarity to glutamate-rich regions of a variety of other proteins. However, the functions of those acidic regions in most such proteins are not known.

The expression of e3 and e22 is temporally regulated. Active expression occurs only during the first few minutes of infection and is then quickly shut off (Fig.16, 20 and 21). Infection with an e3-nonsense mutant phage, SPO1e3m3, yielded no e3 protein, and resulted in an increased and prolonged accumulation of e3 RNA (the much smaller effect in the same direction on e22 RNA may be insignificant), suggesting that transcription of e3 may be autogenously regulated. The small and various sizes of the e3
and e22 RNAs produced during infection suggest active processing of the SPO1 early RNAs (Fig. 21).

Expression of a plasmid-borne e3 gene, in either B. subtilis or E. coli, caused inhibition of host DNA, RNA and protein synthesis, and eventually killed the cell, demonstrating that e3 can cause host-shutoff. To identify the primary target of e3-induced host-shutoff, an e3-resistant E. coli mutant was obtained. Plasmid libraries of this mutant's chromosomal DNA, when screened for genes that could protect wild-type E. coli against e3, yielded the rpoB and dksA genes, which specify the RNA polymerase β subunit and a suppressor for E. coli heat-shock protein DnaK, respectively. The wild-type dksA gene, but not the wild-type rpoB gene, was able to protect against e3, suggesting that the primary e3-resistant mutation was in the rpoB gene and that protection by the dksA gene depended upon overexpression from the plasmid. As outlined in Figure 22, we suggest that e3 shuts off host RNA synthesis and, eventually, DNA and protein syntheses, by specifically inactivating the host RNA polymerase, and that sufficient amounts of a chaperonin-like activity specified by the dksA gene can protect the RNA polymerase against this inactivation.

The activity of the e3 product in phage infection appears to be redundant both for host-shutoff and for phage multiplication. The host-shutoff still occurred normally in infection by the e3 mutant, and it occurred much more rapidly than that caused by expression of the e3 gene in uninfected cells. Therefore, the e3 product must be only one of the components of the host-shutoff machinery, which must include elements whose function is redundant to that of e3. Although at low multiplicity of infection, the mutant SPO1 produced the same amount of progeny as wild-
Figure 22. Cartoon models for mechanisms of various bacteriophage-induced host-shutoffs. *E. coli* phage T7 specifically inactivates the host RNA polymerase by its early gene product 0.7 (T7 produces its own RNA polymerase for phage multiplication) (Geiduschek and Kassavetis, 1988; Hausmann, 1988; Michalewicz and Nicholson, 1992). The early gene *alc* of coliphage T4 interacts directly with the host RNA polymerase and reduces its activity with respect to most host genes. The *alt* and *mod* genes of T4 ADP-ribosylate the α subunit of the host RNA polymerase so as to reduce its activity (Snustad et al., 1983; Kutter et al., 1993). We propose that e3 shuts off host RNA synthesis by distorting the structure of the host RNA polymerase, and that this distortion can be prevented or reversed by a chaperonin-like activity specified by *dksA*. 
type, it gave much higher burst sizes at high multiplicities of infection, suggesting that large quantities of the e3 protein can also be inhibitory to phage growth. Perhaps for that reason, expression of both e3 and e22 was shut off after a brief period of high activity.
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