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Mapping the Genes in the Terminal Redundancy of Bacteriophage SPO1 with Restriction Endonucleases

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Although most early transcription from SPO1, a lytic DNA bacteriophage of *Bacillus subtilis*, is specified by the 12.6-kilobase region of the terminal redundancy, early genes from this region have not been identified by standard genetic means. We mapped genes to DNA regions of the SPO1 terminal redundancy by analyzing in vitro protein synthesis from isolated SPO1 restriction fragments in an *Escherichia coli*-coupled transcription-translation cell-free system. DNA from the terminal redundancy directs the synthesis in vitro of eleven proteins, *e*3, *e*4, *e*6, *e*7, *e*9, *e*12, *e*15, *e*16, *e*18, *e*20, and *e*21, which correspond in mobility on sodium dodecyl sulfate-polyacrylamide gels with authentic SPO1 early proteins. From their mapped positions on the DNA, genes were positioned downstream from most, but not all, of the twelve early promoter regions identified in vitro in the terminal redundancy. The temporal patterns of early protein synthesis in vivo suggest a differential turning on and off of early promoters in the terminal redundancy. Both in vivo and in vitro evidence suggests the existence of previously unidentified early promoter regions upstream from the genes for *e*6 and *e*4 as well as a middle promoter region upstream from the gene for *e*16.

SPO1, a 140-kilobase (kb) DNA bacteriophage of *Bacillus subtilis*, contains a large (12.6 kb) terminal redundancy (TR) which is present as a direct repeat (4). Most SPO1-specific RNA synthesized during the *e* period early in infection (8) hybridizes to restriction fragments from the TR (11, 17, 22). A total of 36 cistrons in SPO1 have been identified by the isolation of conditional mutants (16). Of these, only two cistrons, 27 and 28, both of which map to single copy DNA, have been identified as early genes.

Romeo et al. (19) observed binding of *B. subtilis* RNA polymerase isolated from uninfected cells to 12 early promoter regions, P_E1 through 12 in the SPO1 TR. RNA transcription initiates in vitro at all of these 12 early promoter regions, one of which, P_E1, contains a double promoter (1).

Early SPO1 transcription is characterized as *e* or *em* based on its time of shutoff (8). We have categorized SPO1 early proteins as *e* or *em* using similar criteria (13). Using an *Escherichia coli*-coupled transcription/translation cell-free system (cfs) programmed by SPO1 DNA, we have identified a set of at least 21 SPO1-specific, in vitro-synthesized proteins which correspond in migration on one-dimensional sodium dodecyl sulfate-polyacrylamide gels (SDS-PAG), with early SPO1-specific proteins synthesized in vivo (13). In unpublished experiments many years ago, we had observed, by using 2-min ^{14}C-amino acid pulse labelings at early times after infection, that some SPO1 early proteins are detectable earlier on SDS-PAG than other early proteins. To test whether the observed asynchrony in protein appearance was a result of gene position on polycistronic RNAs, we inhibited RNA polymerase with rifampin at staggered times after infection in the presence of ^{14}C-labeled amino acids. Our results suggested that early protein synthesis in vivo results from utilization of multiple early promoter regions at slightly different times postinfection rather than from translation of long polycistronic messages. The publication of a restriction endonuclease map of the TR of SPO1 (1) and the identification of multiple early promoter regions in the TR (1) allowed us to address the question of the pattern of gene expression in this region.

In this communication we report the mapping of the genes for 11 early SPO1 proteins to regions of the TR. First, genes were mapped to DNA regions of the TR by analyzing in vitro protein synthesis in the *E. coli* cfs from isolated SPO1 restriction fragments. In addition, the locations of genes were deduced from the observed loss of synthesis of their protein gene products in the cfs after cleavage of the DNA at known locations with restriction endonucleases and with BAL31 exonuclease.

Next, based on our analysis of their pattern of in vitro synthesis, we correlated the positions for the genes for early proteins with the promoter regions identified in vitro. Finally, we investigated the pattern of promoter utilization in vivo early in SPO1 infection by analyzing protein synthesis after rifampin poisoning. Our results suggest a differential turning on of early promoters in the TR.

MATERIALS AND METHODS

Bacteria and phage. *B. subtilis* 168 (trp^- Su^-) was used for in vivo studies. When appropriate, cells were UV-irradiated before infection as previously described (13). Wild-type and mutant SPO1 were grown and purified as previously described (20). RNA polymerase was prepared from *B. subtilis* 1005 (Rif^r) as previously described (13). *E. coli* 514 (F^- Δ *lac trp tsx* Str^r) was used in the preparation of the coupled transcription-translation system (10). SPO1-infected extracts were prepared and electrophoresed on SDS-PAG as described (13).

Preparation of SPO1 DNA and restriction digests. Wild-type and mutant SPO1 DNA was prepared as previously described (13). *Eco*RI restriction endonuclease was obtained from Miles Laboratories and used under *Eco*RI* conditions as follows: 40 mM Tris hydrochloride (pH 8.5), 2 mM $MgCl_2$, and 20% glycerol (18). *Eco*RI enzyme was used at 0.4 U/μg of DNA for 4 h. All other enzymes were used at 1 U/μg of DNA under conditions specified by the manufacturer

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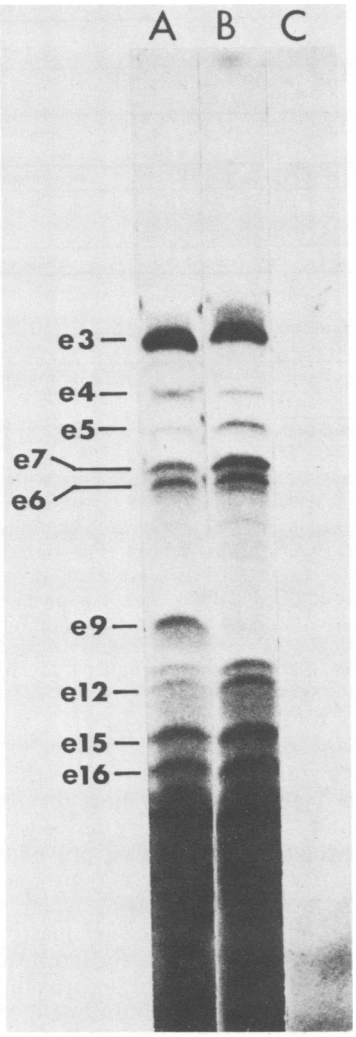

FIG. 1. SPO1-specific protein synthesis in an *E. coli* cfs. ^{14}C-labeled proteins were separated electrophoretically on an SDS-15% PAG and detected by autoradiography. Templates were (A) SPO1 DNA (6,550 cpm), (B) SPO1 DNA digested with *Eco*RI (9,300 cpm), and (C) no DNA (2,100 cpm). Note the lack of early protein *e*9 in lane B. Nomenclature is as previously reported (13). Protein molecular weights (13) are listed in Table 3.

(*Bst*EII, *Bst*NI, *Hae*II and *Msp*I; New England BioLabs, and *Hae*III and *Taq*I; Bethesda Research Laboratories, Inc.).

Restriction fragments were isolated by electrophoresing digested DNA samples into a 1% agarose gel and visualizing them with ethidium bromide; the gel was then sliced with a razor blade. Gel slices were wrapped in Parafilm and frozen at −20°C. Frozen samples were crushed and then thawed, and liquid was squeezed into a siliconized glass tube. Visible agarose was removed by centrifugation. Trace amounts of agarose were removed by extraction twice with buffered phenol and once with chloroform-isoamyl alcohol (24:1). DNA was ethanol precipitated and then suspended in 10 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA. Samples were assayed on agarose gels to determine yield and purity of the fragment.

BAL31 exonuclease digestion. BAL31 was obtained from New England BioLabs. Incubations of restriction fragments with BAL31 were performed under conditions recommended by the supplier as follows: 600 mM NaCl, 12 mM $CaCl_2$, 20 mM Tris hydrochloride (pH 8.0), 1 mM EDTA at 30°C. Typically, 1 μl of enzyme (1.25 U) was used in a reaction volume of 30 μl; 5-μl samples of the reaction mix were removed at the desired times, and the reactions were stopped by mixing with 2-μl samples of 100 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. These samples were then added to cfs reaction tubes on ice and, when the BAL31 digestion series was complete, all cfs tubes were incubated as usual at 30°C for 30 min. We had previously determined that this concentration of EGTA does not affect the cfs.

Autoradiograph scanning. Autoradiographs were scanned using a Gilford spectrophotometer. Radioactivity incorporated into individual peaks was quantitated by calculating the area of the geometric figure (triangle or trapezoid) which gave the best fit for that peak.

RESULTS

In vitro mapping of genes for early proteins to DNA regions of the TR. Although the TR of SPO1 bacteriophage appears to contain most of the genes transcribed heavily early in infection (11, 17, 22), only gene 35, and by inference gene 36, have been mapped to this region of the genome (3). Our laboratory has generated viable SPO1 phage which synthesize mutant forms of several of the early proteins (unpublished data). These results indicate that many of the early SPO1 genes are nonessential and therefore unavailable for mapping by standard genetic means. Therefore, we chose to map the DNA locations for genes in the TR of SPO1 by mapping the DNA regions of the TR which are capable of specifying the synthesis of SPO1 protein gene products in an *E. coli* cfs.

Isolated SPO1 DNA, when added to the *E. coli* cfs, specifies the synthesis of proteins which correspond in mobility on one-dimensional SDS-PAG to most of the early SPO1 proteins synthesized in vivo (13). In addition, we found a few polypeptides synthesized in the cfs from SPO1 DNA which do not correspond in mobility to any authentic SPO1 proteins. Figure 1, lane A, shows protein synthesis in an *E. coli* cfs with isolated SPO1 DNA. The nomenclature of the early proteins is in accordance with Heintz and Shub (13). In the gel system used in this study, the relative mobilities of two proteins, *e*6, and *e*7, are reversed.

Cleavage of SPO1 DNA with restriction endonucleases before use in the cfs results in the selective loss of synthesis of specific proteins. For example, Fig. 1 shows the lack of synthesis of early protein *e*9 from SPO1 DNA which had been cleaved with restriction endonuclease *Eco*RI (lane B). Selective inhibition of the synthesis of early SPO1 proteins by several other restriction enzymes is summarized in Table 1.

Using published restriction maps of the TR of SPO1 as a guide (1), we isolated fragments containing sequences from the TR generated by four restriction enzymes, *Eco*RI, *Bst*NI, *Hae*III, and *Taq*I. The genomic location of each isolated fragment is listed in Table 2 and shown diagrammatically in Fig. 2.

Early SPO1 proteins synthesized from a cfs primed with a given restriction fragment are listed in Fig. 2 within the block designating that restriction fragment. Proteins are listed in an order from left to right which is consistent with their patterns of synthesis from isolated fragments. Additional data supporting the order of genes listed in Fig. 2 was obtained by protein synthesis in the cfs after digestion of whole genome

TABLE 1. Effect of template cleavage on the synthesis of SPO1 proteins whose genes map to the TR

Protein	Fragment directing synthesis[a]						
	*Bst*NI	*Hae*III	*Eco*RI	*Taq*I	*Msp*I	*Hae*II	*Bst*EII
*e*9	1	6	–	8	–	–	+
*e*6	1	–	15	8	–	+	+
*e*4	1	4	15	8	–	+	+
*e*18	1	4	15	8	+	+	+
*e*15	1	4	15	–	–	+	+
*e*3	1	4	26	–	+	–	–
*e*20	7	4	10	(±)	+	+	+
*e*16	7	4	10	–	+	–	–
*e*7	7	4	10	11	+	–	–
*e*21	7	4	10	11	+	(±)	(±)
*e*12	7	4	10	–	+	+	+

[a] A number beneath a restriction enzyme indicates the identity of an isolated restriction fragment which directs the synthesis of a protein in the cfs. +, Continued protein synthesis after cleavage; –, cleavage prevents synthesis; (±), probable continued synthesis.

SPO1 DNA with the other restriction enzymes listed in Table 1 and with BAL31 exonuclease.

Figure 2 also includes the restriction map of the TR (1) for all restriction enzymes used in this study. Also indicated in Fig. 2 are the locations of in vitro-identified promoter regions (1). Placement of genes relative to these promoter regions will be discussed later.

As indicated on Fig. 2, two fragments generated by *Bst*NI endonuclease, *Bst*NI-1 and *Bst*NI-7, which together contain essentially all of the sequences from the TR, produce in the cfs 11 of the 21 identified SPO1 early proteins (13). Isolated fragments from the TR generated by three other enzymes, *Hae*III, *Eco*RI, and *Taq*I, produce subsets of these 11 proteins in the cfs, along with no additional SPO1 early proteins. Since, as shown in Fig. 2 and Table 2, the fragments generated by the four enzymes constitute overlapping sets, it is possible to order unambiguously the positions of the genes for most of the early proteins produced from the

TABLE 2. Map positions for isolated restriction fragments from the TR

Fragment	Coordinates (kb)[a]
*Bst*NI	
1	0.3–8.1
7	8.1–12.6
*Hae*III	
4	0.0–2.45
6	2.45–12.6
*Eco*RI	
20	0.0–2.15
15	2.15–5.1
26	5.1–6.2
10	6.2–12.6
*Taq*I	
8	1.1–4.3
11	9.3–12.3

[a] Map positions are expressed in kilobases from the left terminus of the genome. These values are calculated from data in references 1 and 17, and E. P. Geiduschek, personal communication; and our own data.

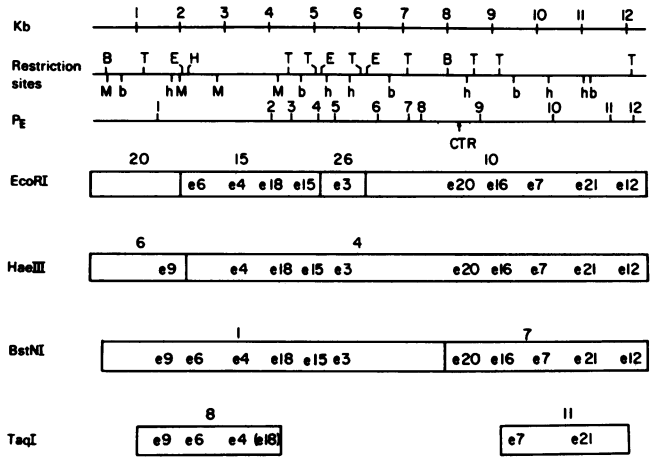

FIG. 2. Synthesis of early SPO1 proteins with isolated SPO1 DNA restriction fragments from the TR. Distances are in kilobases from the left terminus of the genome. Locations of the 12 in vitro-identified early promoter regions (P_E1 through 12) and the central termination region (CTR) are indicated, as well as restriction sites (reference 1; and E. P. Geiduschek, personal communication). The code for restriction enzymes is presented in Table 3. Numbers above boxes indicate the restriction fragments used as templates. Proteins made in vitro from these templates are indicated within each box.

TR. The following discussion will refer to the data summarized in Tables 1 and 2 to show how the order of genes presented in Table 1 and Fig. 2 was derived.

Protein *e*9, whose synthesis was prevented by *Eco*RI cleavage (Fig. 1), was the only SPO1 protein synthesized from restriction fragment *Hae*III-6, which contains sequences from the leftmost end of the genome (0.0 to 2.45 kb). Therefore, the gene for *e*9 must be the leftmost of the SPO1 genes which we detected, and the inactivating *Eco*RI site must be the single *Eco*RI site within *Hae*III-6, located at 2.15 kb. Cleavage of SPO1 DNA with *Hae*II and *Msp*I, which cleave *Hae*III-6 at 1.95 and 2.05 kb, respectively, also prevented *e*9 synthesis.

The gene for protein *e*6 clearly maps adjacent to the gene for *e*9. Protein *e*6, along with *e*4, *e*18, and *e*15, was synthesized from isolated *Eco*RI-15, which maps between 2.15 and 5.1 kb. Since *e*4, *e*18, and *e*15 were synthesized from *Hae*III-4 (2.45 to 12.6 kb), their genes map to the right of the gene for *e*6, whose synthesis was prevented by *Hae*III cleavage at 2.45 kb, the single *Hae*III site in the TR.

Of the proteins synthesized from *Eco*RI-15 (2.15 to 5.1 kb), all except *e*15 were also synthesized from *Taq*I-8 (1.1 to 4.3 kb). Therefore, the gene for *e*15 must be the rightmost gene on *Eco*RI-15 and is inactivated by *Taq*I cleavage at 4.3 kb. We made a similar analysis of gene placement for the remaining proteins synthesized from the TR. Based on these data, the location for the gene for each protein was narrowed down to the DNA region common to all restriction fragments which produce that protein in the cfs (Table 3). For example, the common DNA region for *e*9, which is synthesized from *Bst*NI-1, *Hae*III-6, and *Taq*I-8, extends from the *Taq*I site at 1.1 kb to the *Hae*III site at 2.45 kb.

Table 3 also indicates, for each protein, the effect of restriction enzyme cleavage within the common DNA region. Restriction enzymes which cleave within the common DNA region for a given early protein and whose cleavage does not prevent synthesis of that protein in the cfs are listed

TABLE 3. DNA regions for proteins synthesized in vitro from the terminal redundancy[a]

Protein (mol wt [kDa])	Required coding region (bp)	DNA coordinates (kb)	Common DNA region (bp)	Noninactivating enzymes	Inactivating enzymes
*e*9 (24.5)	669	T 1.1–H 2.45	1,350		h 1.95 M 2.05 E 2.15
*e*6 (30.5)	831	E 2.15–T 4.3	2,150		H 2.45 M (2.8, 4.2)
*e*4 (42.0)	1,143	H 2.45–T 4.3	1,950		M (2.8, 4.2)
*e*18 (12.2)	333	H 2.45–T 4.3	1,950	M (2.8, 4.2)	
*e*15 (16.3)	444	H 2.45–E 5.1	2,650	b 4.7	M (2.8, 4.2) T 4.3
*e*3 (44.0)	1,200	E 5.1–E 6.2	1,100		h (5.3, 5.8) T 6.0
*e*20 (10.8)	294	B 8.1–12.6[b]	4,500	b (9.8, 11.3) h (10.4, 11.2)	
*e*16 (14.9)	406	B 8.1–12.6[b]	4,500		h (8.5, 10.4, 11.2) b (9.8, 11.3) T (8.7, 9.3, 12.3)
*e*7 (29.5)	804	T 9.3–T 12.3	3,000		h (10.4, 11.2) b (9.8, 11.3)
*e*21 (10.0)	273	T 9.3–T 12.3	3,000	h (10.4, 11.2)[c] b (9.8, 11.3)[c]	
*e*12 (19.6)	534	B 8.1–12.6[b]	4,500	h (8.5, 10.4, 11.2) b (9.8, 11.3)	T (8.7, 9.3, 12.3)

[a] Protein molecular weights, expressed in kilodaltons, are from reference 13. DNA coordinates are the ends of the smallest DNA region in common among purified restriction fragments from which each protein has been synthesized in the cfs. Coordinates are measured in kilobases from the left terminus of the genome (Table 2). Inactivation data were obtained from digestions from whole SPO1 DNA. Code for restriction enzymes: E, *Eco*RI; H, *Hae*III; B, *Bst*NI; T, *Taq*I; h, *Hae*II; M *Msp*I; b, *Bst*EII.

[b] Right end of the genome, equivalent to 12.6 kb from the left terminus.

[c] In vitro protein synthesis from SPO1 DNA after *Bst*EII or *Hae*II digestion includes proteins which comigrate on SDS-PAG with *e*21. However, inactivation of *e*21 synthesis by these enzymes could be masked by runoff protein fragments generated from other genes.

under the column heading noninactivating enzymes (Table 3). Numbers indicate the position of site(s) within the common DNA region recognized by each enzyme. Since cuts at these sites do not prevent synthesis of the protein in question, the gene for that protein cannot span any of these sites. Restriction enzymes whose cleavage prevents synthesis of a protein are listed under the column heading inactivating enzymes (Table 3). Numbers indicate the position of site(s) within the common DNA region recognized by each enzyme. When an enzyme cuts at only one site within the region, cleavage at that site must be responsible for inactivating gene expression. When an enzyme cuts at more than one site, cleavage at one or more of the sites could be responsible for inactivation. For example, synthesis of *e*12 from a common DNA region extending between the *Bst*NI site at 8.1 kb to the right end of the TR at 12.6 kb is prevented by cleavage with *Taq*I, which recognizes sites at 8.7, 9.3, and 12.3 kb, but is not prevented by cleavage with *Hae*II (8.5, 10.4 and 11.2 kb) or *Bst*EII (9.8 and 11.3 kb).

Unlike the situation at the left end of the TR, where the gene for *e*9 is obviously the terminal gene, the distribution of restriction sites in the right portion of the TR does not allow for an unambiguous ordering of genes. Therefore, we used BAL31 exonuclease to identify the protein whose gene maps closest to the right end of the genome. Each BAL31 digestion mixture containing an SPO1 restriction fragment was set up as described in the Materials and Methods. At the indicated times, a 5-μl sample of the reaction mix was removed, mixed with EGTA, and then added to a cfs reaction mix tube. Figure 3 shows the effect of BAL31 digestion on subsequent protein synthesis from isolated restriction fragments *Eco*RI-1 and *Eco*RI-10. The extremely low (*Eco*RI-1, 1 min) and high (*Eco*RI-10, 0 min) levels of overall protein synthesis observed in Fig. 3, lanes C and G, respectively, are most likely due to problems of mixing or pipetting. Of more importance are the relative rates of synthesis of individual proteins within each lane. When isolated fragment *Eco*RI-10, the terminal *Eco*RI fragment from the right-hand copy of the TR, was exposed to brief digestion by BAL31 before use in the cfs, its ability to specify synthesis of protein *e*12 was rapidly lost, compared to its ability to specify synthesis of other proteins whose genes also map to *Eco*RI-10. One minute of BAL31 digestion of *Eco*RI-10 (Fig. 3, lane H) dramatically depresses *e*12 synthesis, compared with the high level produced from the intact restriction fragment (lane G). Synthesis of other early proteins are relatively resistant to BAL31 digestion. Con-

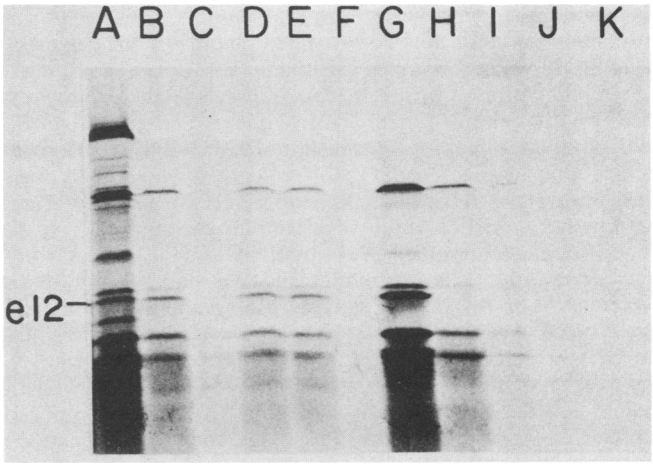

FIG. 3. The effect of BAL31 exonuclease digestion of isolated SPO1 restriction fragments on subsequent protein synthesis in an *E. coli* coupled transcription-translation cfs. Isolated restriction fragments were digested with BAL31. At the indicated times, samples of the reaction were removed, and added to EGTA. Digested and undigested DNA samples were then used as templates in an *E. coli* cfs. ^{14}C-labeled proteins were separated by electrophoresis on an SDS-15% PAG and detected by autoradiography. Lane A, whole genome SPO1 DNA (50,500 cpm); lane B, isolated fragment *Eco*RI-1 (13,000 cpm). Lanes C through F show isolated fragment *Eco*RI-1 subjected to BAL31 exonuclease for the indicated times (lanes): C, 1 min (4,800 cpm); D, 2 min (8,300 cpm); E, 3 min (10,100 cpm); F, 4 min (2,650 cpm). Lane G, isolated fragment *Eco*RI-10 (28,200 cpm). Lanes H through J show isolated fragment *Eco*RI-10 plus BAL31 for the indicated times (lanes): H, 1 min (9,600 cpm); I, 2 min (4,200 cpm); J, 3 min (4,150 cpm). Lane K, no DNA (6,600 cpm). Note the lack of SPO1 early protein *e*12 synthesis after BAL31 digestion of isolated fragment *Eco*RI-10 but not *Eco*RI-1.

versely, synthesis of *e*12 in the cfs from *Eco*RI-1, which contains the equivalent sequences from the left-hand copy of the TR, is not more sensitive to BAL31 digestion than are the other proteins. This indicates that the gene for *e*12 is located nearest to the right end of the TR, and is protected from exonucleolytic digestion in *Eco*RI-1 by the presence of about 14 kb of single-copy DNA (17).

Of the proteins synthesized by *Bst*NI-7 (8.1 to 12.6 kb), two, *e*7 and *e*21, are also synthesized from the internal fragments *Taq*I-11 (9.3 to 12.3 kb). Three proteins, *e*12, *e*16, and *e*20, are synthesized by *Bst*NI-7 but not by *Taq*I-11. *e*12 synthesis is prevented by *Taq*I cleavage (Table 3). Since, as shown by BAL31 digestion (Fig. 3), the gene for *e*12 is located furthest to the right of any gene in the TR, it must be the *Taq*I site at 12.3 kb whose cleavage prevents *e*12 synthesis in the cfs.

The positioning of at least part of the gene for *e*12 to the terminal 300 base pairs (bp) of the genome located to the right of *Taq*I-11 (between 12.3 and 12.6 kb) implies that the other proteins which are produced from *Bst*NI-7 but not *Taq*I-11 (*e*16 and *e*20) are located to the left of *Taq*I-11. Further evidence that this is, in fact, the case will be presented in the discussion of promoter regions.

The data presented thus far permit the following ordering from left to right of the genes for early proteins in the TR: *e*9, *e*6, (*e*4, *e*18), *e*15, *e*3, (*e*20, *e*16), (*e*7, *e*21), and *e*12. The relative positions of genes for proteins indicated within each set of parentheses cannot be determined.

Thus far, we have examined the synthesis of SPO1 early proteins from DNA of the TR in an *E. coli* cfs under the control of endogenous RNA polymerase. We next examined the TR for the presence of genes which could be transcribed in a middle mode in vitro.

Protein synthesis in vitro with gp28-modified RNA polymerase. In vivo synthesis of SPO1 middle RNA classes requires the modification of host RNA polymerase by the product of SPO1 early gene 28 (gp28) (7). In vitro, we have used an *E. coli*-coupled transcription-translation cfs to study SPO1 gene expression by placing SPO1 RNA synthesis under the control of purified *B. subtilis* RNA polymerase isolated from uninfected or SPO1-infected rifampin-resistant cells (21). The procedure involves preincubation of SPO1 DNA with Rif^r *B. subtilis* RNA polymerase. We have found that, whereas *E. coli* Rif^s RNA polymerase competes for binding sites on SPO1 DNA with *B. subtilis* RNA polymerase from uninfected cells, SPO1 gp28-modified *B. subtilis* RNA polymerase recognizes unique sites (21). We (13) have grouped SPO1 proteins into classes analogous to the six temporal classes of RNA first reported by Gage and Geiduschek (8). We found that most early SPO1 proteins which can be synthesized in an *E. coli* cfs display the pattern expected from the products of *e* RNA; they are not synthesized in a cfs under the control of gp28-modified polymerase, and their synthesis is not enhanced after polymerase modification in vivo (13). We did find, however, a few proteins that are synthesized in a pattern consistent with their translation from *em* RNA; they are synthesized in a cfs under the control of gp28-modified polymerase, and their synthesis appears to be enhanced by polymerase modification in vivo.

Absolute identification of the patterns of synthesis of several individual early proteins was complicated by possible middle proteins which comigrate with them on our gel system. It was of interest, therefore, to assay isolated restriction fragments from the TR for protein synthesis in a cfs under the control of gp28-modified RNA polymerase.

Of the four *Eco*RI-generated fragments from the TR, only

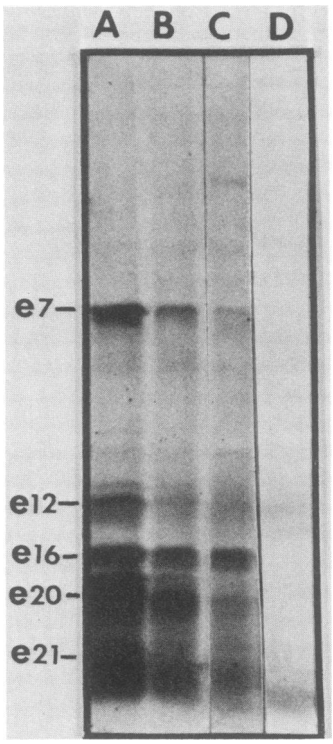

FIG. 4. Effect of RNA polymerase modification on protein synthesis in vitro from an isolated SPO1 restriction fragment from the TR. Equal amounts of isolated SPO1 restriction fragment *Eco*RI-10 were used as templates in an *E. coli* cfs under the control of endogenous *E. coli* RNA polymerase or added *B. subtilis* RNA polymerase. Lane A, endogenous *E. coli* polymerase (15,400 cpm). In lanes B and C DNA was preincubated with Rif^r *B. subtilis* RNA polymerase as previously reported (13) and then added to an *E. coli* cfs in which endogenous Rif^s *E. coli* RNA polymerase had been poisoned with rifampin. Lane B, Rif^r *B. subtilis* RNA polymerase from uninfected cells (7,000 cpm); lane C Rif^r *B. subtilis* RNA polymerase from cells infected with SPO1 mutant *sus*F14, which allows the first but not the second SPO1-specified RNA polymerase modification (3,950 cpm). Lane D, *E. coli* polymerase, no rifampin, no added DNA (7,600 cpm). ^{14}C-labeled proteins were separated on an SDS-15% PAG. An autoradiogram is shown. RNA polymerase preparation was done as previously described (21).

*Eco*RI-10 was active in specifying protein synthesis in a cfs containing modified polymerase. Figure 4 shows protein synthesis from isolated *Eco*RI-10 in a cfs under the control of endogenous Rif^s *E. coli* polymerase, added Rif^r *B. subtilis* holoenzyme, and gp28-modified polymerase isolated from *Rif^r B. subtilis* infected with SPO1. In the cfs under the control of gp28-modified polymerase, *e*16 is selectively synthesized, relative to the other SPO1 proteins encoded by *Eco*RI-10. Protein *e*12, whose synthesis is not favored by gp28-modified polymerase (Fig. 4, lane C) is produced only weakly in a cfs under the direction of *B. subtilis* holoenzyme (lane B) compared with the endogenous Rif^s *E. coli* polymerase (lane A). This may suggest that *E. coli* and *B. subtilis* RNA polymerases have different relative affinities for the various SPO1 early promoters, and that in the case of *e*12 in a rifampin-poisoned cfs, nonproductive initiations by endogenous Rif^s *E. coli* polymerase prevent productive initiations by Rif^r *B. subtilis* polymerase.

Identity of proteins produced in vivo and in vitro. In our analysis thus far, we have assumed that comigration of an in vitro-synthesized protein with an in vivo-synthesized protein

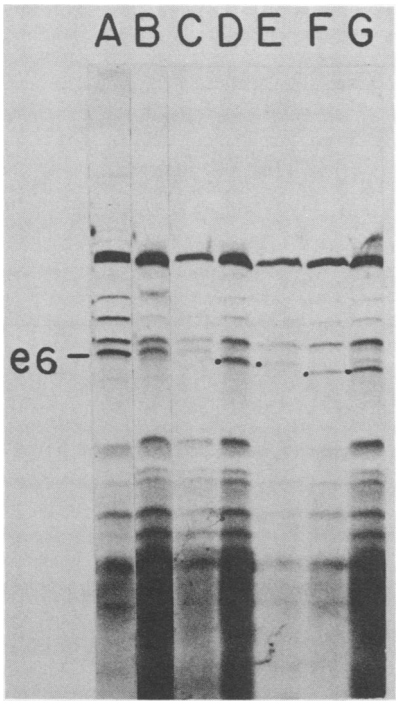

FIG. 5. Comparison of early SPO1 protein synthesis in vivo and in vitro from wild-type and mutant phage. In vivo, UV-irradiated cells were infected with the indicated phage and incubated for 4 min in the presence of ^{14}C-amino acid mix, 1 μCi/ml. In vitro, SPO1 DNA from mutant or wild-type phage was used as template in an *E. coli* cfs. To decrease differences in protein and salt concentrations between in vivo and in vitro samples, 20 μl of in vivo samples or 5 μl of in vitro samples diluted with 15 μl of unlabeled in vivo extract were loaded in each lane. An autoradiogram of an SDS-15% PAG is shown. SPO1 mutant *sus*F21 contains a lesion in gene 28, whose gene product is required for the first modification of *B. subtilis* RNA polymerase by SPO1 (7). All SPO1-specific proteins synthesized by *sus*F21 are therefore early proteins. BR66 and IK7 are SPO1 mutants generated in our laboratory by hydroxylamine mutagenesis (12). (A) In vivo wild-type SPO1 (7,600 cpm); (B) in vitro wild-type SPO1 DNA (6,550 cpm); (C) in vitro *sus*F21 DNA (4,000 cpm); (D) in vitro BR66 DNA (7,400 cpm); (E) in vivo BR66 (2,400 cpm); (F) in vivo, IK7 (2,350 cpm); (G) in vitro IK7 DNA (6,500 cpm). The abnormal positions both in vivo and in vitro of bands in BR66 and IK7 are indicated by dots to the left of lanes D through G. Wild-type position for early protein *e*6 is indicated on the left of the figure.

on a one-dimensional SDS-PAG indicates that the two proteins are identical. In our laboratory we have generated two types of data which support this assumption. First, SPO1 early protein *e*3 synthesized in vivo and from isolated *Eco*RI-26 in vitro produce strikingly similar peptide patterns (6; N. Heintz, Ph.D. thesis, State University of New York at Albany, 1979) when analyzed by the method of Cleveland et al. (2). Second, by hydroxylamine mutagenesis (12), we have generated SPO1 variants which synthesize early proteins of altered mobility when assayed on SDS-PAG. When DNA isolated from these variants is used to direct protein synthesis in an *E. coli* cfs, proteins of the same altered mobility are produced. In vivo and in vitro protein synthesis from two SPO1 variants are displayed in Fig. 5.

One of these variants, IK7, apparently synthesizes a mutant form of early protein *e*6, whose gene maps to restriction fragment *Bst*NI-1 (Table 1). Purified fragment *Bst*NI-1 from wild-type SPO1 specifies production of *e*6 of normal mobility, whereas *Bst*NI-1 from variant IK7 specifies production of a protein of altered mobility (Fig. 6).

Timing of promoter utilization in vivo. Having identified the SPO1 early proteins whose genes map to the TR, we determined the pattern of expression of these genes during infection and correlated this pattern with the mapped positions of the genes. Through pulse-labeling of proteins with ^{14}C-amino acids we have analyzed the time course of SPO1 protein synthesis in cells infected with wild-type SPO1 and with a variety of mutants. We have categorized SPO1 proteins into classes based on their time of synthesis and the mutations which prevent or allow their synthesis (13).

Early proteins are defined as those proteins whose synthesis does not require RNA polymerase modification by gp28. All proteins synthesized during infection by *sus*F21, which is defective in gene 28, are therefore early proteins. However, we have observed by pulse-labeling that initiation of synthesis of the various early proteins during *sus*F21 infection is not synchronous (unpublished data). The observed delay in appearance of some early proteins could be due to at least three different causes: (i) synchronous initiation of protein synthesis, but different lengths of time required for detectable protein to accumulate, (ii) the positioning of genes for delayed proteins near the end of long

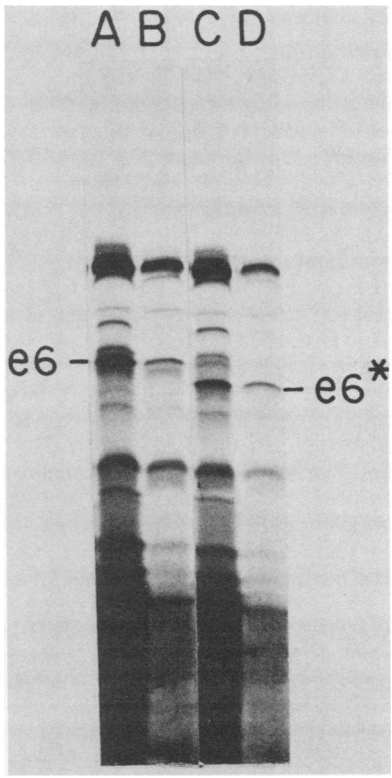

FIG. 6. Comparison of an early SPO1 protein synthesized in vitro from a restriction fragment derived from wild-type or mutant phage DNA. Proteins labeled with ^{14}C-amino acids in an *E. coli* cell-free system were separated electrophoretically on an SDS-15% PAG. An autoradiogram is shown. (A) DNA from wild-type SPO1 digested with *Bst*NI (14,600 cpm), (B) isolated *Bst*NI-1 restriction fragment from wild-type SPO1 (5,600 cpm), (C) DNA from SPO1 mutant IK7 digested with *Bst*NI (12,400 cpm), (D) isolated *Bst*NI-1 restriction fragment from IK7 (2,600 cpm). Note the shift in electrophoretic mobility of SPO1 early protein *e*6 in mutant IK7 compared to wild type.

transcriptional units, or (iii) transcription from different promoters which first form productive initiation complexes with RNA polymerase at different times postinfection. For purposes of this study, a productive initiation complex for a given protein is a rifampin-resistant initiation complex capable of producing a transcript which can be translated into that protein.

To distinguish among these possibilities, we used ^{14}C-amino acids to label proteins synthesized in UV-irradiated cells during the first 10 min of infection with either wild-type SPO1 or *sus*F21. Prior irradiation of cells with UV light was necessary to reduce background of host protein synthesis. Rifampin was added to parallel infected cultures at 0.5-min intervals from 1 to 5 min postinfection to prevent further initiations by RNA polymerase. All cultures were incubated for a total of 10 min to allow elongation of RNA chains and subsequent protein synthesis. Cells were harvested, and samples were prepared for electrophoresis and run on SDS-PAG as described (13).

Synthesis of each protein was quantitated by scanning the autoradiograph in a densitometer. Amount of synthesis of five early proteins whose genes map to the TR could be quantitated. For each protein, density of labeling was plotted against time of rifampin addition (graph not shown). The best straight line for each protein was calculated by using a least-squares fit procedure. Correlation coefficients for all lines are at least 0.99. Since accumulation of protein is a linear function of the length of time of productive polymerase initiation (delay in addition of rifampin), the X-intercept of each line measures the threshold, or earliest time of productive initiation for each protein. The extrapolated times of onset of synthesis from wild-type SPO1 infections were *e*15, 1.5 min; *e*3, 1.7 min; *e*6, 2.0 min; *e*4, 2.6 min; *e*7, 3.0 min. Similar results were obtained from *sus*F21 infection.

Effect of polymerase modification on the synthesis of early proteins in vivo. Of the early proteins whose genes map to the TR, *e*16 is the only protein whose synthesis is favored in a cfs under the control of gp28-modified *B. subtilis* RNA polymerase (Fig. 4). This suggests that *e*16 is an *em* gene, i.e., that it is transcribed both by unmodified and gp28-modified polymerase (13).

To investigate the effect of polymerase modification in vivo on the synthesis of proteins whose genes map to the TR, we pulse-labeled cells infected with *sus*F21 or wild-type SPO1. Synthesis of each protein was quantitated as described in the preceding section. Pulse-labelings for both *sus*F21 and wild-type SPO1 were conducted under two conditions of infection which we had utilized previously to distinguish between proteins synthesized in an *e* or *em* mode (13).

Under the first set of conditions (A) SPO1-specific proteins were labeled from 0 to 10 min after infection of UV-irradiated cells. (Prior irradiation of host cells is necessary to allow visualization of phage-specific proteins). Since the course of SPO1 development is slowed down significantly in UV-irradiated cells compared to unirradiated cells, the 10-min labeling pulse corresponds roughly to the early period of protein synthesis and does not include middle proteins.

Under the second set of conditions (B) to assay protein synthesis during the middle period of infection, SPO1-specific proteins were pulse-labeled between 10 and 14 min postinfection. (Since SPO1 shuts off host protein synthesis before 10 min postinfection, prior UV irradiation of host cells is not required.)

For wild-type SPO1 infection, the relative levels of early proteins observed under the two labeling conditions A and B provide a rough approximation of the relative rates of synthesis (condition A) early in infection, before polymerase modification, and (condition B) at a time when at least some molecules of RNA polymerase are modified by association with gp28. Since *sus*F21 is mutant in gene 28, all protein synthesis under both conditions A and B during *sus*F21 infection is due to host polymerase. Therefore, differences observed in the relative rates of protein synthesis in *sus*F21 and wild-type SPO1 infection are a direct result of lack or presence, respectively, of modified polymerase.

TABLE 4. Synthesis of early SPO1 proteins from *sus*F21 and wild-type SPO1 phage under two conditions of infection

Phage and condition (cpm)[a]	Protein synthesis (ratio)[b]							
	*e*3/*e*5	*e*4/*e*5	*e*6/*e*5	*e*7/*e*5	*e*9/*e*5	*e*12/*e*5	*e*15/*e*5	*e*16/*e*5
*sus*F21								
A (7,400)	2.15	0.49	1.29	0.92	0.53	TD[c]	0.48	—[d]
B (5,700)	0.51	0.61	0.97	0.77	0.53	—	—	0.01
Wild type								
A (6,200)	2.18	0.49	1.37	1.05	0.62	0.21	0.48	—
B (12,900)	0.63	0.56	1.19	1.07	0.62	—	—	0.17

[a] See text for the conditions used.
[b] Equal volumes (20 μl) were separated by SDS-15% PAGE. The autoradiograph was scanned, and density of labeling for each early protein in each lane was quantitated. Synthesis of each protein was normalized to *e*5 synthesis in the same lane.
[c] TD, *e*12 was synthesized in *sus*F21 under condition A, but the band was too diffuse to quantitate.
[d] —, No detectable synthesis.

Table 4 shows the relative amount of ^{14}C-labeled amino acids incorporated into early proteins during infection by *sus*F21 or wild-type SPO1 labeled under both conditions. To provide an internal control, in each case ^{14}C-incorporation into each protein from the TR is normalized to incorporation into *e*5, a prominent early protein (13) whose gene maps to single-copy DNA (N. Heintz, Ph.D. thesis).

*e*16 is the only early protein whose synthesis is favored by polymerase modification (condition B, *e*16/*e*5 = 0.01 for *sus*F21 infection; 0.17 for wild-type SPO1 infection) (Table 4). Infection by *sus*F14, which permits the first but not the second polymerase modification gives an intermediate level of *e*16 (data not shown). Interestingly, the level of *e*16 synthesis is very sensitive to prior UV irradiation of host cells, and *e*16 synthesis is undetectable under condition A for both *sus*F21 and wild-type SPO1 infection.

Aside from *e*16, the levels of synthesis for all other proteins whose genes map to the TR were unaffected by polymerase modification (Table 4). With each phage, some proteins (*e*4, *e*6, *e*7, and *e*9) show approximately equal levels of synthesis, relative to *e*5, under conditions A and B, whereas synthesis of other proteins (*e*3, *e*12, and *e*15) is decreased or absent under condition B.

DISCUSSION

In this study we mapped the genes for 11 early SPO1 proteins with a combined DNA coding requirement of about 6,900 bp to regions of the 12.6-kb TR through the use of restriction enzymes and an *E. coli*-coupled cfs. We presented evidence from the use of hydroxylamine-generated SPO1 mutants that one polypeptide, *e*6, whose gene has been mapped in vitro does, in fact, correspond to its comigrating in vivo-synthesized counterpart.

E. coli RNA polymerase has been shown to faithfully recognize promoters from a wide variety of gram-negative and -positive bacteria, including *B. subtilis* (5, 23), although with different relative efficiencies compared with *B. subtilis* RNA polymerase. In particular, it has long been known that SPO1 DNA can act as a template for asymmetric transcription by *E. coli* RNA polymerase (9). Sequenced early SPO1 promoters correspond closely to the canonical *E. coli* promoter sequence and are recognized strongly by *E. coli* RNA polymerase (15). Since *B. subtilis* is apparently more stringent both in recognition of transcriptional and translational initiation signals than is *E. coli* (15), there exists the possibility of spurious protein production from SPO1 DNA in the *E. coli* cfs.

In addition to the 11 proteins here reported, we found four polypeptides synthesized from defined subregions of the TR of SPO1 in the *E. coli* cfs which do not correspond in mobility to in vivo-synthesized early SPO1 proteins (data not shown). It is unclear whether these additional proteins result from transcriptional or translational anomalies.

Using rifampin in vivo, we have analyzed the time of first polymerase initiation leading to the synthesis of several of the proteins whose genes map to the TR. We have also examined the effect of gp28-modified polymerase on synthesis of the proteins both in vitro and in vivo.

We will now correlate our in vitro-derived gene placement and the pattern of protein synthesis which we observe in vivo with the positions of in vitro-identified promoters in the TR.

Romeo et al. (19) reported 12 regions in the TR which bind *B. subtilis* RNA polymerase holoenzyme tightly enough to form complexes visible in the electron microscope. The positions of these polymerase binding sites, which they designate P_E1 through 12 are indicated on Fig. 2. Using purified *B. subtilis* holoenzyme and nucleotide triphosphates, Brennan et al. (1) observed transcription which started at all 12 promoter regions. Transcription from the first eight promoter regions (P_E1 through 8) proceeds from left to right, whereas transcription from the remaining four promoter regions (P_E9 through 12) proceeds from right to left. Transcription from both directions ends in a small common termination region between P_E8 and P_E9. (In addition, several partial termination regions were observed.)

Placement of early proteins by promoter regions. The two restriction fragments generated from the TR by *Bst*NI cleavage, *Bst*NI-1 (0.3 to 8.1 kb) and *Bst*NI-7 (8.1 to 12.6 kb) contain essentially all of the sequences of the left and right, respectively, transcriptional arms of the TR. We report the synthesis of six proteins (*e*9, *e*6, *e*4, *e*18, *e*15, and *e*3) from *Bst*NI-1 and five proteins (*e*20, *e*16, *e*7, *e*21, and *e*12) from *Bst*NI-7. The mapping of proteins synthesized from *Bst*NI-1 (left transcriptional arm) to subregions of the DNA by other restriction enzymes (Tables 1 and 3, Fig. 2) are consistent with the placement of their genes downstream from the following promoters: P_E1, *e*9, *e*6, *e*4 (*e*18); P_E2, *e*15; P_E3, (*e*18); P_E4, no proteins; P_E5, *e*3; P_E6, 7, and 8, no proteins. Similarly, the mapping of proteins synthesized from *Bst*NI-7 (right transcriptional arm) are consistent with the placement of their genes downstream from the following promoters, arranged from upstream to downstream within the right transcriptional arm: P_E12, *e*12; P_E11 or P_E10, *e*21, *e*7; P_E10, *e*16; P_E9, *e*20.

The above assignments of genes to promoter regions have taken into account the sizes of DNA required to code for each protein (see Table 3). In no case has it been necessary to postulate the existence of overlapping genes. Protein *e*3 poses a problem. Migration of *e*3 on SDS-PAG indicates a molecular weight of 44,000 or a 1,200-bp coding region. We found full-size *e*3 synthesized in vitro from *Eco*RI-26 (1.1 kb). Investigators both in this laboratory (N. Heintz, Ph.D. thesis) and elsewhere (14) have found the polymerase binding site on *Eco*RI-26 at the same location as P_E5 (1), about 200 bp from the left end of the fragment, indicating that the gene for *e*3 can be no larger than 900 bp. An alternative explanation might be that the gene for *e*3 overlaps P_E5 and that transcription from *Eco*RI-26 results from nonspecific or end-specific initiation rather than from initiation at a bona fide promoter. Since the high level of *e*3 synthesis in vitro is unaffected by *Eco*RI digestion (see Fig. 1), we favor the explanation that the gene for *e*3 maps in its entirety downstream from P_E5, and that the *e*3 protein has an anomalously slow migration on SDS-PAG gels, perhaps due to unusual amino acid content.

In the left transcriptional arm, we cannot distinguish between the placement of *e*18 downstream from P_E1 and P_E3. Our data are consistent with the possibility that *e*18 may consist of two proteins of similar molecular weight, whose genes are located downstream from P_E1 and P_E3, respectively. In the left transcriptional arm, we find at least four promoters, P_E4, P_E6, P_E7, and P_E8, which are not followed by genes for early proteins that are detectable on our gel system. The function of these promoters (19) is at present unknown.

In the right transcriptional arm, the placement of restriction sites is such that we cannot distinguish between P_E11 and P_E10 as the most proximal upstream promoter for the genes for *e*21 and *e*7. Protein *e*16, whose synthesis is prevented by *Taq*I cleavage at 9.3 kb, is definitely downstream from P_E10.

Evidence for additional promoter regions. The gene for *e*6 maps to the common DNA region between the *Eco*RI site at 2.15 kb and the *Taq*I site at 4.3 kb (Table 3). Its synthesis in the cfs is prevented by *Hae*III cleavage at 2.45 kb. Therefore, the gene clearly maps in the interval between P_E1 (1.4 kb) and P_E2 (4.0 kb). Surprisingly, protein *e*6 is synthesized in the cfs from isolated restriction fragment *Eco*RI-15, which contains the gene for *e*6, but separated from P_E1, the only identified upstream promoter region. This sugests that transcription in the cfs is initiating at a previously unidentified promoter region. Similarly, the gene for *e*4 also maps to the region between P_E1 and P_E2. Protein *e*4 is synthesized in vitro from isolated *Hae*III-4 (2.45 to 12.6 kb). Since *Hae*III cleavage at 2.45 kb prevents synthesis of *e*6 but not *e*4, the gene for *e*4 must map downstream from the gene for *e*6, and at least some of the polymerase initiations leading to *e*4 synthesis must occur downstream from the site(s) of initiation leading to *e*6 synthesis, suggesting the existence of a second previously unidentified promoter region. An alternative explanation, as mentioned in the case of *e*3, is end-specific initiation of transcription upstream from the genes for *e*6 and *e*4 after *Eco*RI or *Hae*III digestion of SPO1 template.

By staggered addition of rifampin at early times after infection, we calculated the earliest time of productive polymerase initiation leading to the synthesis of the various proteins whose genes map to the TR. Proteins translated from RNA which is initiated at the same promoter should show the same extrapolated initiation times. Proteins *e*6 and *e*4 show slightly different extrapolated initiation times (2.0 and 2.6 min, respectively), supporting the possibility that their RNAs may come from different promoters. Synthesis of *e*9, whose gene maps more proximal to P_E1 than do the

genes for *e*6 and *e*4, could not be quantitated in the autoradiographs from the rifampin addition experiments. However, in two-min pulse-labeled studies both with *sus*F21 and with wild-type SPO1, *e*9 synthesis is typically observed only after *e*6 synthesis has begun (data not shown). This argues against P_E1 as the only polymerase initiation site for RNA leading to *e*6 synthesis.

Proteins *e*15 and *e*3, whose genes map downstream from P_E2 and P_E5, respectively, show earlier times of initiation (1.5 and 1.7 min) than do any of the proteins whose genes map downstream from P_E1. This clearly indicates that transcription of the left transcriptional arm of the TR cannot occur in vivo solely as one long transcriptional unit initiating at P_E1 and supports the utilization of P_E2 and P_E5 for initiation of transcription leading to the synthesis of *e*15 and *e*3, respectively.

The only protein mapping to the right transcriptional arm whose time of initiation could be calculated in the rifampin experiment is *e*7. The extrapolated time of first initiation for *e*7 is 3.0 min, later than any of the times calculated for proteins whose genes map to the left transcriptional arm. We do not at present understand the biological significance of the apparent graded turning on of promoters indicated by the rifampin data.

Evidence for a middle promoter in the TR. Our data, both in vitro and in vivo, point to the existence of a middle promoter in the TR upstream from the gene for *e*16. In vitro, *e*16 is the only SPO1 protein encoded by the TR which is preferentially synthesized by gp28-modified *B. subtilis* RNA polymerase in an *E. coli* cfs (Fig. 4). In vivo, *e*16 is the only SPO1 early protein which is synthesized in higher amounts in wild-type SPO1 infection than in *sus*F21 infection (Table 4). After these observations were made, we learned from E. P. Geiduschek that his group has identified and sequenced a binding site for gp28-modified polymerase downstream from P_E10 (S. Brennan, Ph.D. thesis, University of California, San Diego, 1982).

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