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Multiple Procaryotic Ribonucleic Acid Polymerase Sigma Factors

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INTRODUCTION

Transcriptional specificity in procaryotic organisms resides in the interaction between ribonucleic acid (RNA) polymerase (RPase) holoenzyme and the promoter site of genes and operons. The RPase subunit which controls the specificity of this interaction has been shown to be the sigma (σ) factor, since the RPase core itself does not recognize promoter sites (6). This is not to imply, however, that the other subunits of the core do not have any role in the specificity of the interaction once the holoenzyme has been formed by the addition of σ to the core enzyme. Besides the σ factor, the base sequence in the promoter site also determines whether fruitful interaction will occur between the RPase and deoxyribonucleic acid (DNA), since transcription is initiated only at discrete promoter sites in the genome.

Our concept of the transcription machinery of the procaryotic cell has been influenced by the earlier work done with *Escherichia coli* in which one RPase holoenzyme was identified that appeared to control the expression of all of the genes in the cell. The *E. coli* holoenzyme consists of a core polymerase containing the four subunits ($\alpha_2\beta\beta'$) (5) and the σ^{70} (the superscript stands for the molecular weight of the σ factor $\times 10^{-3}$) (7). Thus it appeared that σ^{70} allowed the holoenzyme to recognize all promoters of *E. coli*, sometimes with the aid of positive factors (109). One early concept to

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explain the specific transcription of bacteriophage genes in *E. coli* proposed the synthesis of a phage-specific σ factor, which would displace the *E. coli* σ factor and change the specificity of the enzyme so that only phage promoters would be recognized (138). However, no biochemical evidence was obtained for a phage-specified σ factor. The search for phage-specific σ factors did lead to the discovery of the T7 phage RPase which was encoded in an early gene of T7 and which specifically transcribed the middle and late genes of T7 (11). However, this phage-specified enzyme function was completely dissociated from any functions of the host RPase holoenzyme and definitely did not act as a phage-specified factor. Thus, although the concept of multiple σ factors interacting with the *E. coli* core was postulated and evidence was sought for new specificity factors, the presence of more than one holoenzyme in *E. coli* was difficult to establish.

In the past several years through studies on phage-infected and uninfected *Bacillus subtilis* cells, evidence has been found for several other RPase holoenzyme forms besides the major RPase holoenzyme $E\sigma^{43}$ (26, 81) (core polymerase [E] + σ^{43} = $E\sigma^{43}$; this enzyme was previously designated as $E\sigma^{55}$ with the molecular weight of the σ factor based on its electrophoretic mobility; sequencing data now indicate that its molecular weight is approximately 43,000 [49]). Results show that phage SPO1-specific factors do displace the host σ^{43} factor and interact with host core enzyme and change its promoter specificity. Also, the uninfected host cell has at least six forms of holoenzymes in which six different factors are associated with the core enzyme. These studies arose from the fact that *B. subtilis* phage-infected cells and *B. subtilis* cells underwent a series of temporally regulated biochemical changes during phage replication and during sporulation. An enzymatic analysis of the RPase of phage-infected cells and sporulating cells revealed the presence of RPase core-associated polypeptides that were normally absent from uninfected or nonsporulating cells. Thus the first evidence for the presence of multiple σ factors emerged from these studies.

In coming full circle on this phenomenon, it is now apparent that *E. coli* cells also contain at least three different factors. In both organisms there is one major σ enzyme (in terms of relative amounts of holoenzyme) and a number of minor σ enzymes that usually comprise <5 to 10% of the total RPase in the cell. The various holoenzymes also recognize distinct promoter sequences and control the expression of various sets of genes.

In this paper we review the evidence for the presence of multiple RPase σ factors, describe their template specificities, compare their properties, summarize the genetics of the σ-factor genes, and discuss their roles in regulating transcription of families of genes. It is apparent that finding the new σ factors adds further complexity to an already complex picture concerning the regulation of gene expression. However, the multiplicity of σ factors also lends support to models which should be helpful in explaining temporal and sequential gene expression during development, not only in procaryotes, but also in eucaryotes. Multiple σ factors of procaryotes are somewhat analogous to the multiple nuclear factors involved in differential gene expression in eucaryotes, except eucaryotic nuclear factors appear to bind to DNA instead of to RPase. Furthermore, when one analyzes the types of genes controlled by the minor σ factors of the cell, the findings suggest that minor enzymes control the expression of genes which are required during stressful situations for the cell, e.g., nutritional deprivation, heat shock, DNA damage, etc. This suggests the possibility that these σ factors and their cognate genes evolved to protect the cell against deleterious growth conditions.

Other reviews pertinent to this subject have been presented by Raibaud and Schwartz (109), McClure (86), Doi (22–24), Losick and Pero (81), Losick (80), Yura and Ishihama (154), and Rosenberg and Court (111).

DEFINING AND ISOLATING NEW σ FACTORS

What are the criteria for designating an RPase core-associated polypeptide as a σ factor? These have been defined by Chamberlin (10) as follows: (i) the protein should be purified and shown to be chemically distinct from the core subunits, as well as showing some constant stoichiometric relationship to the core subunits when associated with the core polymerase; (ii) the protein should be required for some aspect of selective transcription in vitro and reconstitution of the putative σ factor and core polymerase should give an enzyme which possesses a new specificity (i.e., it should recognize a different promoter sequence from that recognized by another holoenzyme); (iii) a corresponding function for the protein should be demonstrable in vivo, which usually entails mapping of its genetic locus and obtaining conditional mutations which affect its functions.

Basically three approaches have been utilized to identify new σ factors: (i) enzymological isolation of new RPase holoenzymes; (ii) use of DNA templates with promoters that are not recognized by the major RPase as a probe to find new RPase activities in vitro; (iii) a genetic approach in which a positive regulatory gene for transcription is found and its product is identified as a σ factor.

These approaches have succeeded because phenomena involving significant levels of differential gene expression were being investigated. In addition, in many cases these studies showed that both a sequential and a temporal regulation of gene expression was occurring and that sets of genes were being turned on. For instance, during replication of phage SPO1 three sets of messenger RNA (mRNA) were identified during early, middle, and late stages of infection (41, 112), and during bacterial sporulation different sets of mRNA could be identified by hybrid competition studies between mRNA isolated from different stages of sporulation (125).

Although these types of regulation could also be explained by other known control mechanisms, such as cyclic adenosine 5′-monophosphatase (cAMP)-catabolite gene activator protein (CAP) activation, removal of repressors or derepression of genes, and synthesis of new RPases (109), evidence was obtained initially with *B. subtilis* phages SPO1 and SP82 and subsequently with sporulating *B. subtilis* cells that new RPase core-associated polypeptides were present. Similar studies with *E. coli* phage T4 initially did not provide conclusive biochemical data for a sigma factor, although phage genes necessary for expression of middle and late genes were identified (38).

In the best documented cases for the occurrence of new factors, the new holoenzyme has been purified and the presence of a new core-associated polypeptide has been demonstrated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis; the putative new sigma factor has been purified and added to the core polymerase, and the resulting holoenzyme has shown a different promoter specificity from the major holoenzyme of the cell. The genetic locus of the new σ factor gene has been determined also. In some cases mutations in the σ gene have been identified.

MULTIPLE RPase HOLOENZYMES IN *B. SUBTILIS*

B. subtilis Phage SPO1 σ Factors

Although it was assumed initially that procaryotic organisms contained only one RPase holoenzyme form that could transcribe all of the genes in a procaryotic genome, it is now likely that most or all procaryotes will contain multiple RPase holoenzyme forms containing different σ factors.

The best evidence for the occurrence of multiple RPase holoenzymes was obtained in studies with two closely related *B. subtilis* phages, SPO1 and SP82, which contain hydroxymethyluracil instead of thymine in their DNAs. These studies will be examined in detail, since they present the type of information which fulfills the criteria for calling a polymerase core-associated protein a σ factor.

Phage SPO1 gene 28 product (σ^{gp28}). Is SPO1 gene 28 product a phage-specified σ factor? SPO1 and SP82 are lytic phages of *B. subtilis* (43). Upon infection of the host the early-stage mRNAs can be made in the absence of protein synthesis by the major host RPase $E\sigma^{43}$ (44, 112), but the synthesis of middle- and late-stage mRNAs requires the expression of early gene 28 and middle genes 33 and 34 (38, 41). There is a clear indication of sequential expression of phage genes at these three stages as measured by mRNA synthesis (41, 116) and the requirement for specific genes which control the expression of subsequent developmental genes (38).

Enzymological analysis of the RPase of phage-infected cells revealed the presence of several new polypeptides associated with the core polymerase (27, 30, 37, 103, 117, 118, 127) ranging in molecular weight from 13,000 to 85,000. These RPase preparations were able to transcribe the middle genes of SPO1 as determined by hybrid competition studies between in vitro synthesized RNA with in vivo middle-stage mRNA for specific sites on the light and heavy strands of phage DNA. The heavy strand was transcribed primarily during the middle and late stages of phage development and could therefore be used to distinguish the transcripts made at these stages (i.e., from middle and late genes) from those made from the light strands during the early stage (i.e., from early genes). In addition, the transcription of a specific middle gene, i.e., the gene for deoxycytidine 5′-monophosphate deaminase, was demonstrated in a coupled transcription-translation system, using SPO1-modified RPase (127).

Since the initial enzymological studies had shown that several different polypeptides were associated with the core polymerase from SPO1-infected cells, the question arose whether any of these polypeptides had the properties expected for a σ factor. By enzyme purification studies (27, 29, 30, 103, 117), the core-associated protein which gave rise to specific transcription of middle genes was found to have a molecular weight of about 26,000 to 28,000. In early dissociation and reassociation studies it was shown that the association of a 28,000-dalton protein with polymerase core enzyme induced template-selective transcription and asymmetric synthesis of SPO1 middle RNA (29). Recent reassociation studies have shown that purified gene 28 product is able to confer specificity for middle gene promoters upon *B. subtilis* and even *E. coli* core polymerase (1, 18).

Finally, it was demonstrated that regulatory gene 28 of SPO1 coded for a polypeptide of about 26,000 daltons (36). This polypeptide is missing from RPase of cells infected with a nonsense mutant of gene 28, *susF21* (37). When the nonsense mutation was suppressed by two *B. subtilis* nonsense suppressors (*sup-1* and *sup-3*), phage replication was regained, with *sup-3*-suppressed phage reproduction being very temperature sensitive relative to *sup-1*. This indicated that different amino acids were being inserted by the two suppressor strains. An investigation of the RPase from these phage-infected, suppressed strains revealed that differently charged variant molecules of gene 28 product were produced during suppression.

If the gene 28 product is a σ factor, then it should confer selective transcription at the DNA binding and RNA chain initiation steps of RNA synthesis. Kinetic studies with the SPO1-modified RPase indeed demonstrated this to be the case (28). In addition, it was shown that the modified RPase discriminated against heterologous DNA and promoters recognized by *B. subtilis* $E\sigma^{43}$.

Another property of σ factor is that, during the early elongation phase of transcription, the σ factor is released from the DNA-RNA-enzyme ternary complex. When the nascent RNA chain is less than 10 nucleotides long, the σ factor is released from the ternary complex and reassociated with another RPase core to initiate a new round of transcription (58). This type of σ factor cycling has been observed with σ^{43} (12, 118) and gp28 (12). Thus gp28 also undergoes a classic σ-like cycling effect during transcription.

These biochemical and genetic data in toto indicated for the first time that a phage-specific σ factor, designated σ^{gp28}, was able to associate with the host core polymerase and change the specificity of the enzyme so that it could transcribe middle genes of SPO1. Furthermore, it conferred all of the enzymatic characteristics associated typically with core-associated σ factor.

If σ^{28} does indeed confer promoted specificity to core polymerase, then an analysis of the early and middle gene promoters should reveal differences in their composition. Since the transcription of the early genes did not require protein synthesis after infection, it was likely that the host $E\sigma^{43}$ was responsible for early gene expression. By restriction and transcription mapping (102, 130, 131), it was found that DNA fragments near the termini of the SPO1 genome contained promoters for early genes. The general location of one of the early promoters was determined by transcription runoff studies with $E\sigma^{43}$ and by sequencing the 5′ end of the runoff transcript (76) and matching the sequence with the DNA sequence near the putative promoter. Upstream from the 5′ end of the transcript were located two regions with high base homology with the consensus −10 and −35 regions of *E. coli* σ^{70} promoters (Table 1). Thus the SPO1 early promoter was recognized by $E\sigma^{43}$ and had conserved −10 and −35 regions similar to that of *E. coli*. Moreover, it was shown that the SPO1 early promoter could be utilized by *E. coli* σ^{70} enzyme in vitro. In comparing the activities of σ^{43} and σ^{70} enzymes with SPO1 early promoter and the *lacUV5* promoter (an *E. coli* promoter), the σ^{43} enzyme showed a clear preference for the SPO1 promoter (a *B. subtilis* promoter) over the *lacUV5* promoter by about 200 times, whereas the σ^{70} enzyme preferred the SPO1 promoter only 10 times more than the *lacUV5* promoter (76).

To determine the properties of the middle gene promoters, a set of SPO1 DNA fragments was obtained by restriction endonuclease digestion. These fragments promoted specific transcription and stable binary enzyme-DNA complex formation by the phage-modified RPase, but not by unmodified host polymerase (130). The source of these DNA fragments was shown to be in the region of the genome which contained middle genes. The general location of the promoters on these DNA fragments was found by runoff transcription studies, by formation of polymerase-fragment binary com-

TABLE 1. Conserved regions of procaryotic promoters

Holoenzyme[a]	−35 region[b]	Spacing (bp)	−10 region	Reference(s)
B. subtilis				
$E\sigma^{43}$ (11)	TTGACA	17–19	TATAAT	91; Wang and Doi, unpublished data
$E\sigma^{37}$ (5)	AGGATTTNA	11–15	GGAATTNTTT	67; Wang and Doi, unpublished data
$E\sigma^{32}$ (2)	AAATC	14–15	TANTGNTTNTA	67
$E\sigma^{29}$ (4)	TTNAAA	14–17	CATATT	67
$E\sigma^{28}$ (2)	CTAAA	16	CCGATAT	48
SPO1$E\sigma^{gp28}$ (5)	TNAGGAGANNA	15–16	TTTNTTT	75
SPO1$E\sigma^{gp33-34}$ (5)	CGTTAGA	17–19	GATATT	67
E. coli				
$E\sigma^{70}$ (168)	TTGACA	16–18	TATAAT	59
$E\sigma^{32}$ (6)	TNTCNCCCTTGAA	13–15	CCCCATTTA	19
T4$E\sigma^{gp55}$ (4)			TATAAATA	15
Klebsiella and *Rhizobium* spp.				
$E\sigma^{gpnifA}$ (12)	CTGGYAY	5	TTGCA	2 ,21, 101

[a] Total number of sequences analyzed for each class of promoters is shown in parentheses.
[b] For T4$E\sigma^{gp55}$, there is no apparent consensus sequence in the −35 region; for $E\sigma^{gpnifA}$ the upstream region is in the −20 instead of the −35 region.

plexes, and by deoxyribonuclease "footprinting" experiments with the gp28 RNA polymerase associated to the DNA fragment. The exact start point of RNA synthesis was determined by sequencing the 5′ end of the transcript (made from the putative promoter), which was then matched with the DNA sequence. The five gp28 promoters that were characterized in that fashion all contained two sequences that were highly conserved in the −10 and −35 regions (75, 132) and that were significantly different from those found for the early SPO1 and *B. subtilis* vegetative genes (Table 1). Thus, these results supported the concept that different σ factors conferred different promoter specificity to the enzyme.

In these in vitro transcription studies it was found that cloned DNA containing thymine instead of hydroxymethyluracil could also serve as a template for early gene transcription, but the transcription of the middle genes by $E\sigma^{gp28}$ required the presence of hydroxymethyluracil and not thymine, since cloned DNA containing thymine was not a suitable template (76). Therefore, under conditions of in vitro transcription 5-hydroxymethyluracil-containing promoters were required.

The SPO1 gene coding for σ^{gp28} has been cloned and sequenced (17). The location of gene 28 on a specific DNA fragment was determined by coupled transcription and translation experiments with cloned SPO1 DNA fragments (12, 17). These experiments led to the isolation of a 1.2-kilobase (kb) *Sau*3A fragment which contained gene 28. This was confirmed by marker rescue experiments with the *Sau*3A fragment in which the fragment contained in a plasmid was introduced in a linear form into a nonpermissive host strain. This strain was then infected with a gene 28 nonsense mutant phage. Recombination between the two genes produced a wild-type SPO1 genome and allowed plaque formation on a lawn of the nonpermissive host (17).

Sequence analysis of the *Sau*3A fragment revealed only a single open reading frame in either direction large enough to encode a polypeptide of about 26,000 daltons, the predicted molecular weight of gp28 from gel electrophoresis (17). The 220-codon open reading frame had a translational initiation codon, ATG, preceded by a ribosomal binding site [ΔG = −14.6 kcal[61.1 kJ]/mol] and coded for a protein with a molecular weight of 25,707. The N-terminal amino acid sequence of gp28 matched perfectly the sequence predicted from the nucleotide sequence. The gene was preceded by a weak early promoter.

σ^{gp28} is a highly charged protein with 17.7% acidic and 15% basic residues. The protein has a high content of secondary structure as predicted by the method of Chou and Fasman (14), with about 70% of the protein in the σ-helix conformation. *E. coli* σ^{70} has about 50 to 55% α-helix content (7).

Although σ^{gp28} interacts with *B. subtilis* and *E. coli* core enzymes and enables both *B. subtilis* and *E. coli* core enzymes to recognize and initiate transcription from SPO1 middle gene promoters (1, 18), it has little or no amino acid homology with the major σ^{43} factor of *B. subtilis* or with σ^{70} of *E. coli* (Table 2). Despite large differences in size, one might expect the σ factors to have a common structure that would allow them to bind to the core enzyme in a similar way. Since there is a distinct lack of amino acid homology between σ^{gp28} and the host σ^{43}, the recognition may depend on other factors such as conformation of the molecule.

Phage SPO1 gene 33 and gene 34 products. Are SPO1 gene 33 and gene 34 products also phage-specified factors? The expression of late SPO1 genes is dependent on the functions of SPO1 genes 33 and 34 (38, 41), which are middle genes. When RPase was isolated from late infected cells, the enzyme contained two new polypeptides with molecular weights of 24,000 and 13,500 (104). This enzyme in the presence of another polypeptide called the delta factor (103) was able to preferentially transcribe late mRNA in vitro from the SPO1 genome (104).

To test their putative σ activity, the 24,000- and 13,500-dalton proteins were dissociated from RPase that had been isolated from late infected cells and then reassociated with polymerase core enzyme. In the presence of both proteins and the delta factor, the reconstituted enzyme was able to

TABLE 2. Sequence survey of procaryotic transcription factors[a]

Homologous σ factors	Reference	Nonhomologous σ factors	Reference(s)
Bs σ^{43} (*rpoD*)	49	Bs SPO1 σ^{gp28}	17
Bs σ^{29} (*spoIIG*)	123	Bs SPO1 σ^{gp33}	16
Bs σ^{22} (*spoIIAC*)	34	Bs SPO1 σ^{gp34}	16
Ec σ^{70} (*rpoD*)	8	Bs φ29 gp4	32
Ec σ^{32} (*rpoH htpR*)	11	Bs DNA primase (*dnaE*)	144
		Ec DNA primase (*dnaG*)	8, 116
		Ec rho factor (*rho*)	106
		Ec NusA protein (*nusA*)	65
		Ec σ^{60} (*ntrA*)	64
		T7 RNA polymerase	120
		Kp σ^{gpntrA} (*ntrA*)	88
		St σ^{gpntrA} (*ntrA*)	S. Kustu, personal communication

[a] Little or no homology is found in the "nonhomologous σ factors." Abbreviations: Bs, *B. subtilis*, Ec, *E. coli*, Kp, *Klebsiella pneumoniae*, St, *Salmonella typhimurium*.

transcribe selectively from the heavy strand of phage SPO1, the strand from which late mRNA is transcribed in vivo (136). Hybrid competition studies with late in vivo mRNA indicated that the enzyme was transcribing from late genes in vitro. Thus the two polypeptides in the presence of delta factor allowed the enzyme to recognize late promoters.

Genetic studies were carried out to determine whether genes 33 and 34 were in fact coding for the two core-associated polypeptides. By use of two-dimensional gel electrophoresis, the 24,000- and 13,500-dalton proteins were identified in phage-infected cells. The analysis of proteins from cells infected with nonsense mutants of gene 33 (*susF14*) and gene 34 (*susF4*) showed that these proteins were missing. By use of suitable suppressor strains, it was found that genes 34 and 33 coded for polypeptides with molecular weights of 24,000 and 13,500, respectively (35). Thus the genetic data indicated that genes 33 and 34 actually coded for the core-associated polypeptides.

To isolate and clone genes 33 and 34, marker rescue experiments were carried out to localize the two genes on the SPO1 genome. Subclones of restriction fragments were used to rescue the infections of nonsense mutations in genes 33 and 34 until the location of the genes was determined more precisely. Finally, by use of a suitable expression vector, it was shown that *Eco*RI* fragment 21 coded for two proteins with molecular weights of about 24,000 and 12,000.

Sequencing studies showed that genes 33 and 34 overlapped by 4 base pairs (bp), with the *opa-1* termination codon of gene 33 overlapping the initiation codon of gene 34: -A-T-G-A- (16). Gene 33 codes for a 101-residue, 11,902-dalton protein and gene 34 codes for a 197-residue, 23,677-dalton protein. gp33 contains 23% basic and 21% acidic residues, while gp34 contains 19.7% basic and 16.8% acidic residues. The amino acid sequences of gp33 and gp34 had no homology to each other (16), to gp28 (17), φ29 P4 factor (32), and *E. coli* σ^{70} (7), or to the major *B. subtilis* σ^{43} factor (49) (Table 2). Thus no sequence homology has been found between the SPO1 σ factors with any other σ factors.

The sequence of late promoters has been shown to contain −10 and −35 regions which are different from those found in middle and early promoters (67) (Table 1). Thus the early, middle, and late SPO1 promoters are recognized by three different holoenzyme forms, each with their own promoter specificities. The major difference for the late enzyme is that two proteins work synergistically with the core enzyme to transcribe late promoters, whereas the early and middle promoters are recognized by holoenzymes with only a single σ factor.

Displacement of host σ by phage. How do the phage-specific σ factors displace the host σ^{43} from the core enzyme? The displacement of σ^{43} by phage-specific σ factor plays an important role in the successive expression of middle and late genes. The question of whether sigma factors compete with each other for the core enzyme was studied by Chelm et al. (13). They used an assay which could measure the activity of $E\sigma^{43}$ and $E\sigma^{gp28}$ in the same reaction mixture. The assay was based on transcription of σ^{43}- and σ^{gp28}-controlled SPO1 genes followed by gel electrophoresis and quantitation of their transcripts. It was found that σ^{43} competed effectively against σ^{gp28} for the core enzyme below 0.2 total ionic strength, while σ^{gp28} was more effective at higher, but still physiological, values. Kinetic studies showed that the formation of holoenzyme was much faster with σ^{gp28} than with σ^{43} and that σ^{gp28} could displace σ^{43} from $E\sigma^{43}$ to form $E\sigma^{gp28}$. Thus these data indicated that conversion of $E\sigma^{43}$ by a displacement reaction could result in the successive formation of a holoenzyme form that was capable of transcribing middle genes. However, the possibility for other mechanisms remains, such as a virus-specified inactivator of σ^{43} factor (13). The conversion of $E\sigma^{gp28}$ to $E\sigma^{gp33,34}$ has not been studied to date.

B. subtilis Vegetative Minor RPase Holoenzymes

RPase core-associated polypeptides. The presence of multiple σ factors during phage replication in *B. subtilis* prompted an analysis of RPase during sporulation of *B. subtilis*, since there was an obvious successive expression of a large number of genes during spore development. Furthermore, the transcription of phage genes was shown to be significantly different in vegetative cells and in sporulating cells (152). One of the earliest ideas concerning the regulation of spore gene expression was postulated by Losick and Sonenshein (82) who thought that the vegetative σ^{43} factor could be displaced by sporulation-specific σ factors to regulate the expression of sporulation-specific genes.

The first studies indicated that large polypeptides were associated with the RNA polymerase core of vegetative or sporulating cells. These core-associated polypeptides had molecular weights of 70,000 to 85,000 (52, 53, 79), 95,000 (99), and 92,000 (66). Subsequently, smaller polypeptides were found associated with the vegetative core with molecular weights of 21,000 (called delta factor) (103) and of 11,000 and 9,500 (27). None of these proteins have been shown to act as σ factors, although the delta factor prevents nonpromoter interactions of RPase with DNA (27, 30, 103) and therefore exerts a higher degree of transcriptional specificity on RPase holoenzyme.

The first small polypeptides associated with RPase core from sporulating cells were reported by Fukuda et al. (40). They reported two polypeptides with molecular weights of about 27,000 and 20,000. The 27,000-dalton protein appeared at $T_{3.5}$ and the 20,000-dalton protein appeared at $T_{5.5}$ during sporulation (the subscript represents hours after the stationary phase of growth has been reached). The ratio of these proteins to the core was 1:1, and the polymerases containing these subunits both eluted much later than the vegetative

$E\sigma^{43}$ enzyme from a DNA-cellulose column (39, 40). The transcriptional activities of the RPase containing these polypeptides were significantly different from those of $E\sigma^{43}$ on several DNA templates, and the sporulation enzyme containing the 27,000 to 29,000 subunit was much more sensitive to inhibition by netropsin than $E\sigma^{43}$ on a *B. subtilis* template (94).

Linn et al. (79) reported the presence of a sporulation-phase RPase containing two additional polypeptides with molecular weights of 85,000 and 27,000. This enzyme was able to bind to phosphocellulose columns more tightly than the vegetative enzyme and differed in its response to Mg^{2+} and KCl. The 27,000 polypeptide appeared to be the same as that reported by Fukuda et al. (39, 40).

Since most of the core-associated polypeptides were smaller in molecular weight than the major σ^{43} factor, there was a possibility that the smaller peptides were derived from σ^{43}. A two-dimensional (148) analysis of the peptides derived by proteolysis of core-associated polypeptides with molecular weights of 37,000, 29,000, 21,000 (delta), 34,000, and 23,000 revealed that no peptide homology existed between these factors and σ^{43}. Therefore, they were not derived by partial proteolysis from σ^{43}, but were products of different genes. A partial peptide homology was observed between σ^{43} and *E. coli* σ^{70}. Furthermore, antibody produced against σ^{43} did not cross-react with any of the smaller core-associated polypeptides, although it did cross-react with *E. coli* σ^{70} (148). Thus, although the minor core-associated polypeptides were derived independently from σ^{43}, it appeared that σ^{43} and σ^{70} had a common ancestor.

These early studies showed the presence of a variety of core-associated polypeptides and fulfilled one of the criteria for σ factors. The proof that the associated polypeptides were truly σ factors required the fulfillment of two other criteria, i.e., demonstration of their promoter specificity and their activity in vivo.

One of the key steps for demonstrating the specificity of a putative σ factor depends on the availability of a DNA fragment containing the putative cognate promoter. Since no sporulation genes had been cloned or specifically identified, Segall and Losick (114) in a series of astute experiments attempted and succeeded in isolating a DNA fragment containing at least two sporulation stage-specific genes. Their procedure was to label mRNA during the sporulation stage with ^{32}P, identify sporulation-specific mRNAs by comparative polyacrylamide gel electrophoresis, identify *Eco*RI-treated DNA fragments which contained this mRNA sequence, clone these DNA fragments in plasmid pMB9, and identify specific clones that contained DNA that hybridized with the mRNA probe. An 0.4-kb relatively stable mRNA was found in $T_{1.5}$ sporulating cells and was used as a probe to identify the DNA fragment which contained a complementary sequence. A 4.4-kb piece of DNA from *Eco*RI-treated *B. subtilis* DNA was found which hybridized with the mRNA probe. The transcription of this gene occurred very early during stage 0 of sporulation, at $T_{0.5}$, and was not expressed in mutants *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H*, which are blocked at stage 0 of sporulation (100). A sporulation mutant blocked at a later stage, stage II, was able to synthesize the 0.4-kb transcript. The isolation of this fragment containing a very early sporulation gene (0.4-kb gene) was a vital step in the characterization of a new σ factor in *B. subtilis*, since the 0.4-kb gene could be used as a specific DNA template to probe for an RPase with a specificity different from that of the major vegetative $E\sigma^{43}$ enzyme.

$E\sigma^{37}$ enzyme of *B. subtilis*. A 770-bp subfragment of the 4.4-kb DNA fragment described above (114) was used as a probe template to test for specific RNA-synthesizing activity in vitro. This subfragment containing the promoter-proximal portion of the 0.4-kb gene was inactive as a template for the major $E\sigma^{43}$ holoenzyme. RPase was purified from early sporulation-stage cells of *B. subtilis*, using the promoter-proximal portion of the 0.4-kb gene as the probe template. An active RPase fraction was eluted from a phosphocellulose column that could utilize the 0.4-kb gene-containing DNA as a template. The RPase in this enzyme fraction contained a polypeptide of 37,000 daltons associated with the core enzyme, and no σ^{43} was present (56, 57). This new form of enzyme, $E\sigma^{37}$ (Table 2), was able to synthesize specific runoff transcripts from the probe template.

To determine whether this 37,000-dalton protein determined the promoter specificity of the enzyme, the protein was dissociated from the core with 6 M urea and separated from the core subunits by phosphocellulose column chromatography. In reassociation studies (57), it was found that the pure 37,000-dalton protein allowed the polymerase core enzyme to specifically transcribe two genes, 0.4 kb and *ctc*, on the 4.4-kb DNA fragment (114). The *ctc* was transcribed with much greater efficiency than *spoVG* (90). The expression of *ctc* was greatly enhanced during the sporulation phase, but its expression was not dependent on *spo0* genes. Thus two sporulation-related genes were found to be transcribed by the form of holoenzyme containing σ^{37}.

$E\sigma^{37}$ has been found in both vegetative and early sporulation cells (26, 56); therefore, it probably functions during growth and early sporulation. Its activity peaks at the end of the log phase of growth, declines at about T_2, and appears to be influenced by the growth medium (80, 100). Also, expression from *spoVG* is deficient in cells with mutations in the early sporulation genes *spo0A*, *spo0B*, *spo0C*, *spo0E*, *spo0F*, *spo0H*, and *spo0K* (100, 155). The expression of the σ^{37} promoter (51, 150; see below) of the subtilisin gene (*aprA*) is also controlled by *spo0* mutations (61). These facts indicate that $E\sigma^{37}$ is regulated in a complex fashion during the onset and very early stages of sporulation.

$E\sigma^{37}$ controls the expression of the extracellular alkaline serine protease (subtilisin) gene (*aprA*) of *B. subtilis* (51, 150). In vitro $E\sigma^{37}$, but not $E\sigma^{43}$, was able to utilize the subtilisin gene promoter (51, 150). This was the first gene with an identified protein product that was shown to be expressed from a minor sigma promoter. This gene is usually expressed after the end of the log phase of growth. It has been known for some time that the appearance of subtilisin was associated with the initial stages of sporulation, since the *spo0A* mutation had a pleiotropic effect which eliminated subtilisin synthesis and blocked sporulation at stage 0 (61, 105). To demonstrate whether subtilisin activity was essential for sporulation, the subtilisin gene was cloned (151), a deletion was made in the gene in vitro, and the deletion was inserted into the chromosome by gene conversion (66). This deletion mutant lacking subtilisin activity was still capable of sporulating. Thus σ^{37} promoters regulate genes involved in both sporulation and the synthesis of at least one extracellular enzyme which is not required for sporulation. These genes appear to be related primarily by their temporally expressed promoters and not by any role in the sporulation process.

Two other σ^{37} promoters have been observed. One is a promoter for a cryptic gene, *P43* (145), and the other is one of three tandem promoters that regulates the expression of the σ^{43} operon (L.-F. Wang and R. H. Doi, unpublished

observation). Both of these σ^{37} promoters are temporally expressed during the early stage of sporulation.

What are the features of σ^{37} promoters? Since the $E\sigma^{43}$ did not utilize the σ^{37} promoters, it was presumed that σ^{37} promoters would be different from the σ^{43} promoters. By use of transcription and S1 nuclease mapping, the promoters for *spoVG* (67), *ctc* (92), *P43* (145), *aprA* (subtilisin) (150), and σ^{43} operon (Wang and Doi, unpublished data) were localized, cloned, and sequenced. They all contained −10 and −35 regions which were quite distinct from those found for the consensus σ^{43} promoters (Table 1).

Deoxyribonuclease footprinting experiments indicated that the $E\sigma^{37}$ binding site extended from 43 to 51 bp upstream from the transcription start point (i.e., −43 to −51) to 22 bp (i.e., +22) downstream from the start point (90). The close contact points between $E\sigma^{37}$ and the cognate promoter of *ctc* was revealed by examining the pattern of purine methylation by dimethylsulfate subsequent to enzyme binding to the promoter (89). The results showed that the methylation of five guanines and four adenines was suppressed or enhanced by the bound enzyme. These bases were clustered in the −10 and −35 regions of the promoter that also contained the conserved homologous sequences. Thus the minor σ^{37} enzyme appears to interact with the promoter in a fashion analogous to that of the major σ^{43} enzyme.

$E\sigma^{32}$ enzyme of *B. subtilis*. The enzyme containing σ^{32} was found when it was observed that transcription from the *spoVG* promoter yielded two transcripts which were initiated at two +1 positions only 10 bases apart (67). The promoter which yielded a transcript that was 10 bases longer, i.e., the upstream promoter (P1), was the σ^{37} promoter discussed in the previous section. The downstream promoter (P2) which yielded the 10-base shorter transcript turned out to be a σ^{32} promoter which was transcribed by $E\sigma^{32}$ which was contaminating the $E\sigma^{37}$ enzyme preparation. The $E\sigma^{32}$ was purified away from $E\sigma^{37}$, and suitable dissociation-reassociation experiments revealed that a 32,000-dalton protein conferred promoter specificity to the core polymerase (67) and directed the transcription of *spoVG* gene from promoter P2. Thus the *spoVG* promoter really consists of two overlapping promoters transcribed by two different forms of RPase holoenzymes.

The σ^{32} promoter has also been found to regulate the expression of *ctc* (67) and the subtilisin gene (*aprA*) (150). The conserved sequences of the σ^{32} promoter at the −10 and −35 regions are different from those for the σ^{43} and σ^{37} promoters (Table 1).

$E\sigma^{28}$ enzyme of *B. subtilis*. The existence of this form of *B. subtilis* holoenzyme $E\sigma^{28}$, came about from studies on the utilization of *E. coli* phage T7 promoters by heterologous RPases from several different species of bacteria. Most of the heterologous RPases recognized the A and C promoters of phage T7 with varying efficiencies when compared with *E. coli* $E\sigma^{70}$. These results supported the idea that the same T7 promoters were recognized by these various heterologous holoenzymes. However, with the *B. subtilis* vegetative cell RPase preparation an additional promoter J activity was shown to be present on T7 DNA which was not recognized by the major $E\sigma^{43}$ enzyme of *B. subtilis* (66) or by any other eubacterial RPase that was tested (146). The T7 DNA was used as a template probe to purify an RPase from a vegetative cell extract of *B. subtilis* (147). This RPase contained a polypeptide with a molecular weight of 28,000. This enzyme had much higher activity on total DNA or certain cloned DNA fragments from *B. subtilis* than with T7 phage, φ29 phage, pBR322, or pHV14 DNA. In reconstitution experiments the addition of the purified 28,000-dalton protein (σ^{28}) restored promoter specificity to the core polymerase (147).

To isolate σ^{28} promoters, $E\sigma^{28}$ was used to synthesize RNA probes by transcribing total *B. subtilis* DNA *Eco*RI and *Hin*dIII fragments (47). These in vitro transcripts were then used to screen a lambda phage library of *B. subtilis* genomic fragments. This method turned out to be a very efficient procedure for detecting σ^{28} promoter-containing plaques. A statistical analysis of their isolates was consistent with the notion that approximately 25 to 30 σ^{28} promoters were present in the *B. subtilis* genome (47).

The sequence analysis of σ^{28} promoters from two cryptic genes revealed −10 and −35 sequences quite distinct from the other σ promoters of *B. subtilis* (48) (Table 1). Both promoters had identical sequences -CTAAA- and -CCGATAT- at their −35 and −10 regions, respectively. One other σ^{28} promoter has been tentatively identified as a heat shock promoter for the σ^{43} gene (*rpoD*) (143; L.-F. Wang and R. H. Doi, *in* A. T. Ganesan and J. A. Hoch, ed., *Genetics and Biotechnology of Bacilli*, vol. 2, in press). It is located in the C terminus of the *dnaE* gene which precedes the *rpoD* in the σ^{43} operon (143, 144).

The expression of some σ^{28} promoters has been shown to be controlled by the nutritional environment of the cell as well as the developmental state of the cell (46). Generally growth in richer medium yielded a higher level of transcripts from σ^{28}-controlled genes, until the medium became so rich that the cells could not sporulate; i.e., sporulation was catabolite repressed. Under these conditions there was a twofold decline of σ^{28} transcripts compared with growth in a sporulation medium.

Two types of data indicated that expression from σ^{28} promoters was under developmental control. A very rapid decline in transcripts from two σ^{28} promoters was observed when wild-type cells reached the end of the logarithmic growth stage. By T_1 (i.e., 1 h after the end of the logarithmic growth phase or during stage 0 of sporulation) these transcripts had been reduced three- to fourfold, and by T_2 virtually none of these transcripts were present. In the second type of evidence, it was found that *spo0* mutations affect transcription from σ^{28} promoters. Mutations in *spo0A*, *spo0B*, *spo0C*, and *spo0F* substantially reduced the level of transcription from σ^{28} promoters, whereas mutations in *spo0H*, *spo0J*, and *spo0K* had no effect on the normal production of transcripts from the two σ^{28} promoters. In contrast to these effects on σ^{28} promoters, the *spo0* mutations had no effects on the σ^{43} *veg* promoter (46). Thus, although the functions of σ^{28} promoter-controlled genes are not known (except for the putative heat-shock-induced σ^{43} gene), many of them are regulated by events occurring during early sporulation. The link between *spo0* gene functions and σ^{28}-controlled expression of genes suggests that σ^{28} promoters may control genes which respond to nutritional conditions of the cell prior to and at the onset of sporulation (46).

Thus, three minor forms of RPase, $E\sigma^{37}$, $E\sigma^{32}$, and $E\sigma^{28}$, have been demonstrated in vegetative cells besides the major $E\sigma^{43}$ enzyme (Table 1). The $E\sigma^{43}$ comprises about 90 to 95% of the total enzyme during growth (26). The role of the minor forms during growth is not well established, and it appears that they become much more active at the end of the logarithmic phase of growth and at the onset of sporulation (80).

The genetic loci for σ^{37}, σ^{32}, and σ^{28} are still unknown, and the final criterion for establishing their σ function has not

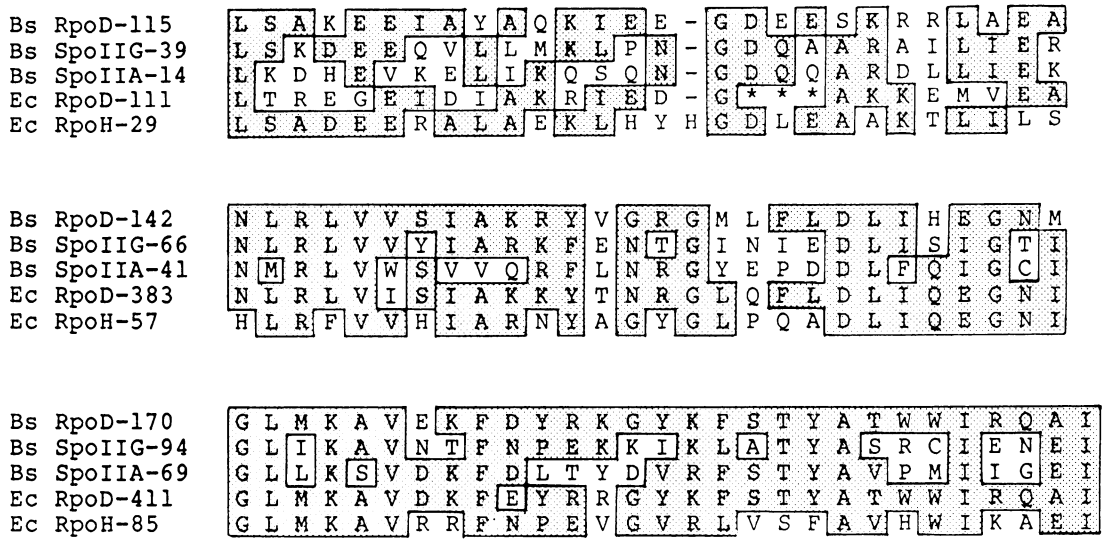

FIG. 1. Homology between various σ factors from *B. subtilis* and *E. coli*. The most homologous region of the σ factors is indicated. See original references for complete sequences. Bs RpoD (σ^{43}), Bs SpoIIG (σ^{29}), and Bs SpoIIA (σ^{22}) are *B. subtilis* factors. Ec RpoD (σ^{70}) and Ec RpoH (σ^{32}) are *E. coli* σ factors.

been satisfied to date. However, the other biochemical evidence strongly indicate that they are σ factors.

B. subtilis Sporulation-Specific Holoenzymes

The *spoIIG* gene product is σ^{29}. The observation of a 27,000- to 29,000-dalton polypeptide associated with RPase core of sporulating cells was the first evidence for a small σ-like factor in *B. subtilis*. The enzyme containing this polypeptide had a very different elution pattern from $E\sigma^{43}$ from a DNA-cellulose column (39, 40, 55); it had a different template specificity from the vegetative $E\sigma^{43}$ enzyme and required the progression of the sporulation process to about stage II for its synthesis (40, 79, 95).

The promoter specificity of the enzyme was determined by use of the cloned 4.4-kb template containing several sporulation-related genes (114). A relative broad activity of the enzyme was noted for several promoters, but one σ^{29}-specific promoter called the L promoter was observed (55). Reassociation studies demonstrated that the purified 29,000-dalton protein was necessary to confer promoter specificity on the core enzyme. Analyses of $E\sigma^{29}$ with the *ctc* promoter have shown that base substitutions in the −35 and −10 regions affect the utilization of the promoter (133, 134), indicating that both regions of the promoter are involved in specific recognition activities. The promoter sequence recognized by σ^{29} contains CATATT-T and A-TT-AAAA at the −10 and −35 regions, respectively (N. E. L. Unnasch, Ph.D. Thesis, Harvard University, Cambridge, Mass., 1982) and is quite different from promoters recognized by the vegetative RPase holoenzymes (Table 1).

The gene for σ^{29} has been found by reverse genetics techniques. The *spoIIG* gene was cloned and sequenced and its deduced product was observed to be partially homologous with *B. subtilis* σ^{43} and *E. coli* σ^{70} (122) (Fig. 1; Tables 2 and 3). The product of *spoIIG* contained 239 amino acids and was calculated to have a molecular weight of 27,652. It is rich in charged amino acids (33.5%) and contains 42 basic and 38 acidic residues. The cloned *spoIIG* was able to correct the Spo^- phenotype of *spoIIG* mutations.

The biochemical evidence showed that *spoIIG* coded for a *spoIIG*-specific RNA synthesized only during sporulation and that anti-σ^{29} antibody cross-reacted with the protein(s) encoded by the cloned *spoIIG* gene (142). Immunological analyses (Western blots) indicated the presence of two proteins, P31 and P29, which cross-reacted with a monoclonal antibody made against σ^{29}. It thus appears that σ^{29} is synthesized initially as a P31 precursor protein and then processed into σ^{29} by removal of about 24 amino acids from the N terminus of P31 (142). The final molecular weight of the protein would then be about 24,000. As with other factors which have been studied, the σ^{29} also appears to have an anomolous electrophoretic mobility in SDS-polyacrylamide gels; e.g., *E. coli* σ^{70} and *B. subtilis* σ^{43} have apparent molecular weights of 86,000 and 55,000 by SDS-gel electrophoresis. Thus the actual molecular weight of the σ factor is usually about 80% of that calculated by use of SDS-gel electrophoretic techniques.

The presence of a σ^{29}-like factor has been observed by immunological techniques in sporulating, but not vegetative cells of several *Bacillus* species (141), and thus this σ factor may be a common transcriptional regulatory factor among sporulating *Bacillus* spp.

The identification of the *spoIIG* gene product as the σ^{29} factor demonstrated for the first time that a specific RPase-associated protein was essential for sporulation. This fact supported the model for the sequential expression of sets of

TABLE 3. Overall sequence homology between sigma factors[a]

Protein	% Homology				
	Bs RpoD	Bs SpoIIG	Bs SpoIIAC	Ec RpoD	Ec RpoH
Bs RpoD		25.9	21.0	58.1	36.3
Bs SpoIIG	16.2		32.8	20.1	29.7
Bs SpoIIAC	11.1	26.8		25.1	27.7
Ec RpoD	57.1	13.2	13.4		34.9
Ec RpoH	27.8	25.0	19.0	26.7	

[a] The 248-residue insert of *E. coli* RpoD was omitted when the comparisons were made. For every pair of proteins compared, two different values were obtained due to their size differences. The larger values are tabulated in the upper right part of the table, and the smaller values are tabulated in the lower left. Bs, *B. subtilis*. Ec, *E. coli*.

sporulation genes by sequentially modified RPase during development (81).

The *spoIIAC* gene product is tentatively identified as σ^{22}. Recent cloning and sequencing studies (34) have revealed that the *spoIIAC* locus codes for a protein with a molecular weight of around 22,000 and a partial amino acid sequence homology with σ^{43} and σ^{29} of *B. subtilis* and with σ^{70} and σ^{32} of *E. coli* (31) (Fig. 1; Tables 2 and 3). The SpoIIAC protein has a molecular weight similar to the core-associated protein isolated by Fukuda et al. (39, 40) from T_{3-5} cells. The transcription of the *spoIIA* locus does not depend on *spoIIG* or on the other *spoII* loci. If the *spoIIAC* gene is shown to code for another sporulation σ factor, it will be the second transcription factor required for stage II of development and the sixth σ factor found in uninfected cells of *B. subtilis*. If one considers the phage SPO1-induced σ factors, there are eight to nine σ factors which can interact with the core enzyme of *B. subtilis*. It appears likely that future studies will result in the identification of more *B. subtilis* σ factors.

Models for Interaction of the *B. subtilis* Multiple Holoenzymes with Their Cognate Promoters

Two ideas have been proposed for the mechanism of interaction of the various holoenzymes with promoters (81): (i) in the first case, it is proposed that the interaction of the different σ factors with the core enzyme results in a conformational change in the core enzyme which allows it to recognize different promoter sequences; (ii) in the second case, since the -10 and -35 regions both are altered in the promoter and it is unlikely that some of the smaller σ factors could make contact simultaneously with both conserved regions of the promoter, it is proposed that the holoenzyme first makes contact with the -35 region in a "closed" complex and then slides to the -10 region to form an "open" complex (10). The second model is currently favored since the presence of about nine σ factors in *B. subtilis* cells makes it unlikely that the enzyme will assume so many different conformations as proposed in the first model.

E. COLI MINOR RPase HOLOENZYMES

Heat Shock Regulatory Protein, HtpR, Is σ^{32}

After the initial discovery of *E. coli* σ^{70} and its role in transcriptional specificity, it appeared that just one form of RPase holoenzyme was capable of transcribing all genes of the *E. coli* genome. The positive regulatory factors required for the expression of some catabolite-controlled operons were shown to be DNA-binding proteins which facilitated the interaction between $E\sigma^{70}$ and its cognate promoters (109).

The discovery of the first new σ factor in *E. coli* came from studies on heat shock protein genes. A positive regulatory gene (*htpR*, *hin*) whose product was required for the active transcription of heat-inducible genes and operons was identified (96, 151). This gene was located at 75 to 76 min on the *E. coli* map. Cloning and sequencing studies revealed that the gene coded for a protein with a molecular weight of 32,381 (74). A comparison of the DNA sequence revealed that seven segments of the *htpR* gene consisting of 15 or more bases had >85% identity with fragments from *rpoD*, revealing a reasonably good degree of homology between the two genes. A comparison of the amino acid sequence homology revealed a 14-amino acid sequence which was identical between the HtpR protein and σ^{70} (74) (Fig. 1; Tables 2 and 3).

Since HtpR had sequence homology with σ^{70}, it was logical to assume that it might be a protein with a sigma-like function and that a minor RPase form may be present in *E. coli*. The purification of a putative heat shock form of RPase was facilitated by the use of a DNA template containing a promoter which controlled the expression of *rpoD* during heat shock (135). Runoff transcripts which were synthesized from the heat shock promoter were used to recognize and purify enzyme fractions which had a specificity for this heat shock promoter. The HtpR product with a molecular weight of around 32,000 copurified with the RPase activity and apparently conferred heat shock promoter specificity to the core polymerase.

To determine whether the HtpR product was functioning as a σ factor, the protein was overproduced by use of a suitable expression plasmid and tested in reassociation studies with core enzyme. The protein conferred specificity to the core and allowed the recognition of two heat shock promoters which regulated the expression of *rpoD* and *dnaK* (54). Two-dimensional gel electrophoresis and double-isotope labeling experiments with minicells containing a recombinant plasmid were used to prove that the cloned *htpR* gene encoded the protein with the same size and charge as the previously identified HtpR (98). Thus the *htpR* gene product had all of the properties expected of a σ factor and has been designated as *E. coli* σ^{32} (54).

The sequence of six heat shock gene promoters has been determined (97) and shows a consensus sequence at the -10 and -35 regions of -CCCCATAT- and -CTTGAA-, respectively (Table 1). These conserved sequences are significantly different from the consensus -10 (TATAAT) and -35 (TTGACA) sequences of σ^{70} promoters.

Enteric *ntrA* Gene Product Is a σ Factor

The enteric bacteria possess a control system which can respond to nitrogen limitation in the growth medium. Genetic analysis has revealed that the activation of nitrogen-regulated (Ntr) genes requires two positive factors, the products of *ntrA* and *ntrC* (42, 87). Since the *Salmonella typhimurium* and *E. coli* Ntr systems have provided essentially identical genetic and biochemical data, the results from both of these organisms will be discussed as an "enteric" phenomenon.

An example of an Ntr gene is *glnA*, which codes for glutamine synthetase. This gene has two promoters (P1 and P2) preceding the structural information of the gene; the downstream P2 requires the *ntrA* product (gp*ntrA*) and the *ntrC* product (gp*ntrC*) for expression, whereas P1 (about 100 bp upstream of P2) is activated by cAMP-cAMP receptor protein (CRP) and does not require the products of *ntrA* or *ntrC* (21, 110).

Biochemical evidence now indicates that transcription from the *glnA* P2 promoter involves activation by the DNA-binding product of gene *ntrC* (*glnG*) and the product of gene *ntrA* (*glnF*) which functions as a σ factor (60, 64). Five closely spaced binding sites (within 110 bp) with dyad symmetry for *ntrC* product have been identified upstream of P2 by deoxyribonuclease footprinting techniques (60). Since sequence analysis of the *glnA* promoter P2 showed unusual features and its expression required both gp*ntrC* and gp*ntrA*, it was thought that the gp*ntrA* might be a new σ factor. Enzyme purification was carried out for RPase activity, using the *glnA* promoter as the template in a coupled transcription-translation system (60). The in vitro purification revealed the presence of a novel form of RPase which

contained a protein which had a molecular weight of 73,000 based on its mobility during gel electrophoresis. This new form of RPase was able to utilize *glnA* P2 but not σ^{70} promoters. The Eσ^{70} was not able to utilize the *glnA* P2 promoter.

When the *ntrA* (*glnF*) gene was fused to a strong *tac* promoter, large amounts of gp*ntrA* were produced (64). The gp*ntrA* was acidic, did not bind to phosphocellulose but did bind to heparin agarose, and had a molecular weight of 75,000 based on its electrophoretic mobility. The size of the protein differed from the molecular weight derived from the length of the gene which was predicted to code for a protein of 60,000 daltons. It is interesting that the actual molecular weight of a σ factor is usually about 0.8 of the molecular weight determined by its electrophoretic mobility in SDS-polyacrylamide gels.

The gp*ntrA* was able to bind to RPase core, and with supercoiled template containing *glnA* P2, no other protein factors were required for specific transcription from this promoter. The linear template, however, was inactive for this form of the enzyme. With the linear template transcription occurred from *glnA* when core enzyme, gp*ntrA*, gp*ntrB*, and gp*ntrC* were added (64). S1 nuclease mapping experiments showed that initiation of transcription from the *glnA* P2 was identical when either in vitro or in vivo RNA was used for these studies.

All these data are convincing that the *ntrA* gene product is a new σ factor. Thus the gp*ntrA* has been designated as σ^{60}, and it has been proposed that the *ntrA* gene be called *rpoN* (64). The σ^{60} is not homologous with the σ^{70} or σ^{32} (Table 2).

The Eσ^{60} probably regulates the expression of the enteric *ntr* and *nif* genes since the promoters for these genes have similar sequences in their −10 and −21 regions (2, 21). These promoters are very different from the promoters for Eσ^{70} and Eσ^{32} (Table 1).

E. coli Phage T4 gp55 Is a σ Factor

During T4 phage development the early genes are transcribed by unmodified *E. coli* RPase from T4 promoters which are undistinguishable from strong *E. coli* promoters. After early infection the host RPase undergoes a number of modifications including adenosine 5′-diphosphate ribosylation and the addition of five phage-encoded polypeptides to the RPase core. Three of these proteins are required for late transcription and are encoded by T4 genes 33, 45, and 55. It was shown that two phage T4 late promoters were transcribed in vitro by RPase from wild type-infected T4 cells, but not by RPase from T4 gene 55 mutant-infected cells (68).

In in vitro analyses of T4 promoter transcription, the addition of anti-σ^{70} antibody inhibited transcription from early promoters, but not from late promoters. These results indicated that Eσ^{70} was not involved in late transcription. In reconstitution studies, late RPase was dissociated and its subunits were separated by electrophoresis. Four protein bands besides the core subunits were evident with apparent molecular weights of 10,000, 15,000, 23,000 (gp55), and 26,000. When these proteins were added to the core enzyme, only the 23,000-dalton protein encoded by gene 55 was necessary for conferring transcription specificity to the core enzyme for late promoters (69). Thus the gp55 is a T4 phage-encoded σ factor (Table 1). The exact function of phage T4 gp33 remains obscure, although a role for blocking σ^{70} binding to the core enzymes has been proposed (69).

Currently four different σ factors are known to interact with the *E. coli* core enzyme: σ^{70} (RpoD), σ^{32} (HtpR), σ^{60} (NtrA), and σ^{23} (T4 gp55) (Table 1). It is likely that other σ factors will be isolated, since the presence of minor σ factors appears to be a general feature of procaryotic metabolism and not related only to developmental systems.

PROMOTER ORGANIZATION OF *B. SUBTILIS* AND ENTERIC ORGANISMS

In virtually every gene or operon which has been studied in bacilli, overlapping promoters, tandem promoters, or a combination of overlapping and tandem promoters have been observed. These complex promoter regions usually consist of promoters with different holoenzyme specificities; e.g., there are regions which contain promoters for Eσ^{37} and Eσ^{32} (67, 150), Eσ^{43} and Eσ^{37} (145), Eσ^{37}, Eσ^{32}, and Eσ^{29} (133), and Eσ^{43}, Eσ^{37}, and Eσ^{32} (150; S. S. Park and R. H. Doi, unpublished data). This allows the gene or operon to be expressed under different nutritional conditions or developmental stages. The presence of multiple promoters may be a common feature for the bacilli.

In the enteric organisms several cases of tandem promoters have also been reported. Many have tandem σ^{70} promoters which may be differentially regulated; e.g., one may be controlled by cAMP-CRP or one may be controlled by stringent conditions. In the case of *glnA* two different promoters, σ^{70} and σ^{60} promoters, are tandemly arranged, with the σ^{70} promoter being under control of cAMP-CRP and the σ^{60} promoter under control of gp*ntrC* (64, 110). There are tandem σ^{70} promoters which control expression of the σ^{70} operon (8, 83, 84) and an internal heat shock σ^{32} promoter (54) which specifically controls the expression of only the *rpoD* (σ^{70}) gene. Tandem promoters have also been reported for the *gal* operon (9), β-lactamase gene of pBR322 (4), and *rrn* operons (20, 45, 153). Thus, it may turn out that multiple promoters and holoenzymes may be more common than formerly recognized even in the enteric bacteria.

HETEROLOGOUS EXPRESSION OF MINOR σ PROMOTERS

Can the minor σ promoters of *B. subtilis* be expressed in *E. coli*? This was tested by cloning the N terminus of the *B. subtilis* subtilisin gene (149) in *E. coli* in a shuttle expression plasmid (50). The expression of the subtilisin gene had been shown to occur from a σ^{37} promoter both in vitro (51) and in vivo (152) in *B. subtilis*. The promoter was also utilized by *E. coli*, and S1 nuclease mapping experiments with in vivo RNA from *E. coli* showed that the same +1 transcription initiation point was being used in *E. coli* as was used in *B. subtilis* (149). At that time it was suggested that either the Eσ^{70} enzyme had a broad promoter specificity or a minor RPase form existed in *E. coli* which was capable of utilizing a *B. subtilis* σ^{37} promoter (149). The existence of two minor forms of RPase in *E. coli* indicates that the second suggestion may be more plausible; i.e., *E. coli* may contain a σ^{37}-like enzyme.

This follows from the fact that two *B. subtilis* σ^{28} promoters are expressed in *E. coli* by Eσ^{32} (the heat shock form of the enzyme) (3). Thus, these two enzymes from two different species have overlapping promoter specificities. What is interesting is that the σ^{28} promoters are expressed in the absence of heat shock in *E. coli*, which indicates that Eσ^{32} is present constitutively and that the heat shock process must induce a transcription activation factor which works in conjunction with Eσ^{32} to express the heat shock genes. Thus, the σ^{32} factor is necessary but not sufficient to express heat shock genes (3).

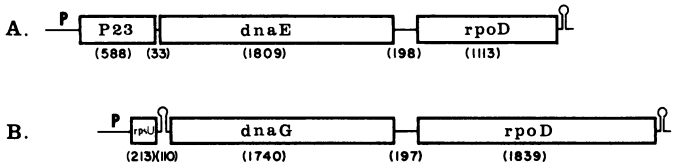

FIG. 2. Schematic representation of the organization of the *B. subtilis* (A) and *E. coli* (B) major σ operons. (A) is from Wang and Doi (143). (B) is adapted from the data for *E. coli* (8, 83). The *B. subtilis* σ^{43} operon (A) contains *P23*, an unidentified gene, *dnaE* (DNA primase), and *rpoD* (σ^{43}). The *E. coli* σ^{70} operon (B) contains *rpsU* (S21), *dnaG* (DNA primase), and *rpoD* (σ^{70}). The numbers in parentheses represent base pairs. The P stands for the promoter region at the 5′ end of the operon. The stem and loop regions indicate transcription termination sites. Other specific regulatory sites are not indicated.

When the promoters for *B. subtilis* $E\sigma^{28}$ and *E. coli* $E\sigma^{32}$ are compared (Table 1), there is a high degree of similarity in their conserved −10 and −35 regions. Thus, it is possible that since a *B. subtilis* σ^{37} promoter was expressed in *E. coli* an enzyme with this type of promoter specificity may also exist in *E. coli* (149).

GENETIC ORGANIZATION OF THE MAJOR σ OPERONS OF *B. SUBTILIS* AND *E. COLI*

To understand the relationships between the various factors within an organism, it will be necessary to understand the genetics of each of the σ factors, the regulation of expression of the σ factors, the mechanisms which determine the relative affinity of the σ factors to the core enzyme, and the physiological and developmental conditions which favor expression of one holoenzyme form over the other. As an initial step to understanding this vast and complex interrelationship, the genetic organization of the major σ factors of *B. subtilis* and *E. coli* has been studied.

E. coli σ^{70} Operon

It was initially demonstrated in *E. coli* that the σ^{70} gene (*rpoD*) existed within an operon with the following composition (8, 83) (Fig. 2): P–*rpsU* (S21)–*dnaG* (DNA primase)–*rpoD* (σ^{70}). This operon maps at 66 min on the *E. coli* map (92, 113) and codes for elements of the translation (S21 is a protein associated with the 30S ribosome), replication (DNA primase), and transcription (σ^{70}) machinery. Obviously the regulation of this operon is of great importance, since the synthesis of the major macromolecules of the cell are affected by the expression of this operon. The regulation is complex since a "high" (*rpsU*), a "low" (*dnaG*), and a "middle" (*rpoD*) expressed gene all exist within the same operon. It is likely that a number of different mechanisms, such as control by ribosome binding strengths, codon usage, mRNA processing, anti-sense RNA, internal promoters, relative mRNA stability at the 5′ and 3′ ends, etc., will be involved.

B. subtilis σ^{43} Operon

The recent analysis of the σ^{43} operon of *B. subtilis* has revealed the following organization (143; Fig. 2): P–*P23* (function unknown)–*dnaE* (DNA primase)–*rpoD* (σ^{43}). This operon maps at about 225° on the *B. subtilis* genetic map (107, 108). This major σ operon is similar to the σ^{70} operon found in *E. coli* in that the last two genes of the operon have identical functions. There is also a high amino acid homology between the products of the second (144) and third (49) genes.

However, there are several significant differences between the two operons. (i) The first gene of the *B. subtilis* operon codes for a protein (P23) with a molecular weight of 23,000. This is much larger than the S21 protein (about 8,000 daltons) coded by the first gene of the *E. coli* operon. (ii) There is no amino acid sequence homology between P23 and S21. This is in sharp contrast to the products of the second and third genes, which show reasonably good homology. (iii) The intercistronic spaces between genes 1 and 2 are quite different for the two operons, although the intercistronic space between genes 2 and 3 are quite similar. Furthermore, there are preliminary data (Wang and Doi, unpublished data) which indicate that the transcriptional regulatory signals differ in their composition and organization.

Genetic Loci of Minor σ Factors

The *ntrA* gene of *E. coli* maps at 68 min (85). In conjunction with two other regulatory genes (*ntrB* and *ntrC*), *ntrA*, which codes for σ^{60}, regulates the expression of a large number of genes involved in nitrogen metabolism (2, 21, 85). It has been proposed that the *ntrA* gene be called *rpoN* (64), since it is a σ factor and part of the transcription machinery.

The *htpR* gene of *E. coli* maps at 75 to 76 min (96, 151). This gene was identified as a conditional temperature-sensitive mutation which abolished the expression of heat shock protein genes of *E. coli* upon temperature shiftup (96, 151). The locus has now been designated as *rpoH* (54) for the heat shock σ factor.

The genetic loci for σ^{37}, σ^{32}, and σ^{28} of *B. subtilis* are still unknown. The genetic loci for σ^{29} (*spoIIG*) and σ^{22} (*spoIIAC*; σ^{22} has been identified tentatively as a sporulation factor [31]) are at about 135° and 210°, respectively (104). Mutations in *spoIIG* and *spoIIAC* block sporulation at stage II. σ^{22} is the third gene of an operon and is preceded by genes coding for proteins of molecular weights 13,100 and 16,300 (34).

MUTANT σ GENES AND THEIR PHENOTYPES

Several mutations have been found in the σ genes. In *B. subtilis*, mutations in σ genes which control sporulation have the dramatic effect of blocking the developmental process at stage II of sporulation. The temperature-sensitive mutation in the *rpoH* gene prevents the expression of heat shock genes. The mutations in the *ntrA* gene prevents proper expression of nitrogen metabolism genes such as glutamine synthetase and also nitrogen fixation (*nif*) genes. Pleiotropic effects of mutations in the major σ genes of *E. coli* and *B. subtilis* have also been noted.

alt (*rpoD2*) and Catabolite Repression

In *E. coli* the *alt* mutation compensated for the loss of the cAMP-CRP system and allowed expression from catabolite-sensitive operons in the absence of enhanced levels of cAMP (115, 139). The *alt* mutation was found to be a mutation in the *rpoD* gene, since the altered properties of the RPase from *alt-1* was shown to reside in the σ^{70} factor (139); this mutation is designated as *rpoD2*. The mutation was localized to a single CGC arginine codon at position 596 in the σ^{70} polypeptide (63). The *rpoD2* mutation increases the expression of the *araBAD* up to 12-fold in the absence of cAMP-CRP, but has no effect on *lac* and reduces expression from *malT*-activated operons. Thus a point mutation in the *rpoD* gene can have profound effects on the expression of catabolite-repressed genes.

crsA (*rpoD47*), Catabolite Repression, and *spo0* Genes

The role of the *B. subtilis* $E\sigma^{43}$ in catabolite repression has been implicated by findings of mutations in the σ^{43} gene (*rpoD*). Takahashi (129) reported mutations in *B. subtilis* which resulted in catabolite-resistant sporulation (*crs*). These Crs mutants were able to sporulate in the presence of high levels of glucose that normally repressed sporulation. The mutations mapped to six independent sites on the *B. subtilis* chromosome (*crsA–crsF*) (126). Two of these sites have been identified as loci which affect the transcription machinery of *B. subtilis*. *crsE* was mapped to the *rpoB-C* locus (127) and *crsA* was mapped to a site within the *rpoD* (σ^{43}) gene (107, 108). Sequence analysis revealed that the *crsA* mutation was actually a two-base change which converted a proline codon (CCT) to a phenylalanine codon (TTT), and the mutation has now been designated as *rpoD47* (67).

This *rpoD47* mutation has a profound effect on the functional capacity of the cell, since it is now able to sporulate in the presence of high levels of glucose and therefore appears to bypass the effects of catabolite repression. Furthermore, this mutation is able to suppress a number of *spo0* mutations which block sporulation at stage 0 (i.e., at the very onset of the sporulation process) (67, 77), and in a reciprocal fashion some *spo0* genes can suppress *rpoD47* (67). These results suggest that σ^{43} or the holoenzyme ordinarily plays a critical role in catabolite repression which is reversed during the onset of sporulation when nutrients are depleted. Since the *spo0* genes may code for nutrient sensor-transducers (46, 100) or transcriptional regulatory factors (33, 122, 137) or both, it appears that the *spo0* gene products directly or indirectly influence the function of RPase. The *rpoD47* mutation may affect the conformation of the σ^{43} or the $E\sigma^{43}$ in such a way that the signals from the *spo0* genes are no longer necessary for the $E\sigma^{43}$ to recognize the promoters of catabolite-repressed genes. Preliminary data with glucose-sensitive promoters indicate that the Crs phenomenon can be observed at the transcription level of individual genes (K. Asada, F. Kawamura, and R. H. Doi, unpublished observations).

Since proteins coded by *spo0A* and *spo0F* have some homology with OmpR and CheY from *E. coli* (33, 122, 137), it suggests the possibility that they are some type of transcription-regulatory factors. The term cosigma has been proposed for ancillary transcription factors that facilitate the activity of holoenzymes (3). Thus the chemotactic and nutrient sensing functions of the cell appear to either directly or indirectly transduce information concerning the environment to the RPase σ^{43} holoenzyme. An idea has been proposed suggesting a linkage among catabolite repression, Spo0 products, and σ^{43} function based on these observations (25, 67).

EVOLUTION OF σ FACTORS

Conserved Sequence Homology

When the amino acid sequence pattern of the various *B. subtilis* and *E. coli* σ factors are compared for homology, two patterns emerge (Fig. 1; Tables 2 and 3). In one case one can recognize the presence of homology between a set of σ factors (Fig. 1; Table 3). These include the *B. subtilis* σ^{43}, σ^{29}, and σ^{22} and the *E. coli* σ^{70} and σ^{32} (Fig. 1).

Among the three *B. subtilis* σ factors which show homology, the percent homology ranges from 11 to 33%, while that between the *E. coli rpoD* and *rpoH* gene products is between 27 and 35% (Table 3).

If the *B. subtilis* σ^{28} is also the heat shock regulatory factor, the σ^{28} will probably have some homology with σ^{43}. This follows from the fact that HtpR is partially homologous to σ^{70} and σ^{43} (Fig. 1).

The highest homology exists between *B. subtilis* σ^{43} and *E. coli* σ^{70} (49; Fig. 1). If one disregards the 248-amino acid sequence missing from the *B. subtilis* σ^{43}, there is about a 58% homology between these two major σ factors (49). The greatest degree of homology within these two molecules exists in the C terminus of these molecules, and it is also within this region that the greatest degree of homology exists between the major and minor σ factors (Fig. 1).

The *E. coli* major σ^{70} is considerably larger than the *B. subtilis* σ^{43}. Although there is homology between the N and C termini of the molecules, the σ^{70} contains a central region which is "missing" from σ^{43} (49). The *E. coli rpoD800* mutations, which is a 14-amino acid in-phase deletion, lies within this missing region (62); therefore, this region does not appear to be necessary for the basic functions of σ^{70}. It is possible that this region is involved in some regulatory aspects.

Lack of Sequence Homology

In another group of σ factors one finds little or no homology between any of them or with any of the factors of the "homologous" group (Table 2). These include the phage-coded factors from *B. subtilis* phages SPO1 (16, 17) and φ29 (32), including gp28, gp33, gp34, and P4, and the *ntrA* factors from a number of enteric organisms (Table 2).

The results with the major σ factors of *B. subtilis* and *E. coli* suggest that a common ancestral σ gene existed before *B. subtilis* and *E. coli* diverged. The smaller minor σ factors which are partially homologous with the major σ factors probably evolved from them. On the other hand, those σ factors which have no homology with the major σ factors may have evolved from the major σ factors and changed so much that no degree of homology is now existent or they evolved independently from their ancestral σ genes and therefore lack any homology with them.

In phage SPO1 there is no homology among all three of the phage-encoded σ factors with σ^{43}, nor is there any homology among themselves (16, 17); thus, even within this small genome, it appears that the three phage-specified σ factors evolved quite independently.

Sequences and Functions

All σ factors, however, must have at least two common functional sites: (i) a binding site for attachment to the core enzyme to form the holoenzyme and (ii) an activity site for formation of the open complex during the initiation of transcription. The complete lack of sequence homology between several σ factors which interact with the same core enzyme indicates that this core recognition site is not dependent on a specific amino acid sequence but probably on a conformational property of the σ factors. Since free σ factor can bind to DNA (72, 73, 121), it appears that a DNA recognition or binding site is present on the σ factors. The presence of this type of site in DNA-binding proteins has been noted (see reference 49 for summary). It has been proposed that the highly conserved regions of the major σ factors are involved in sigma binding to the core (124); however these conserved sequences are not found in all σ

factors. Therefore, other factors besides specific sequences such as conformation of the σ factor are probably involved in the binding of σ to core enzyme.

The promoter recognition site of the σ factors or the holoenzymes must differ, since each form of holoenzyme recognizes different −10 and −35 sequences. The association of a large number of different σ factors with the same core enzyme indicates that the specificity of each of the holoenzymes resides more in the σ factors than in some conformational modification resulting from the σ-core interaction (81). In the most favored model of promoter recognition it is thought that each σ factor recognizes sequentially its cognate −35 region during closed complex formation and its cognate −10 region before forming the open initiation complex (81).

PHYSIOLOGICAL FUNCTIONS OF THE MINOR RPase HOLOENZYME

Regulation of Genes Expressed During Environmental Stress

The minor RPase holoenzymes appear to control genes that respond to stressful situations that confront the procaryotic cell, genes involved in developmental processes, and nonessential genes. Examples of deleterious environmental conditions include the following: (i) nutrient deprivation (the cell responds in many ways, including increased motility and chemotactic responses, release of genes from catabolite repression, activation of nitrogen metabolism genes, secretion of extracellular degradative enzymes); (ii) heat shock (heat shock genes are expressed); (iii) chemical shock (ethanol can turn on several of the heat shock genes); (iv) DNA metabolism genes (DNA damage induces the expression of the several DNA repair-type functions).

Although it has not been shown that all genes which control responses to environmental insults are under the regulation of minor σ enzymes and promoters, several examples have been discussed above that demonstrate that minor σ enzymes are involved in cellular responses to environmental changes or stimuli.

Regulation of Developmental and Nonessential Genes

The second role of minor σ factors appears to be in their control of developmental genes. The best examples to date are illustrated with *Bacillus* phage SPO1 and *E. coli* phage T4 development and the sporulation and sporulation-associated functions of *B. subtilis*. The minor σ enzymes regulate the sequential expression of sets of genes that control the ordered morphological development of phage SPO1 and spores. This is one of several possible mechanisms used in phage morphogenesis since other types of transcriptional control have been reported, e.g., in phage T7 and lambda development (see reference 22 for review).

The minor enzymes appear to control the expression of many nonessential genes. For instance, minor enzymes control the expression of extracellular enzyme genes and sporulation genes which are not necessary for the growth of bacilli. It is possible that catabolite-repressed genes and genes for the synthesis of secondary metabolites, including antibiotics, proteinacious inclusions, and toxins that are synthesized after growth has ceased, may also be under control of minor promoters.

At least one minor enzyme controls the expression of heat shock genes which code for essential genes; however, their expression occurs from at least two promoters, usually a major promoter and a special heat shock promoter. Thus they are a special case of essential genes being transcribed from both a minor and a major promoter.

CONCLUSIONS

It is now established that the procaryotic transcriptional machinery has a complex system of multiple RPase holoenzymes consisting of several different σ factors that interact with a common core enzyme. Each holoenzyme form can interact specifically with a cognate promoter that has a unique consensus sequence in its −35 and −10 regions. It appears that factors have evolved either from a common ancestral σ factor or from independent events that led to proteins which could interact with core enzyme and acts as σ factors. The minor σ enzymes appear to control genes that are generally nonessential and that evolved to allow the cell to respond to various environmental stresses. The other major role of minor σ enzymes is to express genes in a sequential fashion during developmental processes in procaryotic cells. It is highly likely that other minor σ factors will be reported in a variety of procaryotes.

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