Activation of Cell-Specific Transcription by a Serine Phosphatase at the Site of Asymmetric Division

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Cell fate is determined by cell-specific activation of transcription factor σ^F after asymmetric division during sporulation by *Bacillus subtilis*. The activity of σ^F is governed by SpoIIAA, SpoIIAB, and SpoIIE, a membrane protein localized at the polar septum. SpoIIAB binds to and inhibits σ^F, and SpoIIAA inhibits SpoIIAB, which prevents SpoIIAB from binding to σ^F. SpoIIAB is also a serine kinase that inactivates SpoIIAA. Here, it is demonstrated that SpoIIE dephosphorylates SpoIIAA-P and overcomes SpoIIAB-mediated inhibition of σ^F. The finding that SpoIIE is a serine phosphatase links asymmetric division to the pathway governing cell-specific gene transcription.

The σ^F factor of the bacterium *Bacillus subtilis* is a transcriptional control protein that is required for the establishment of cell-specific gene expression during the process of sporulation (*1*). During sporulation, a polar septum forms that partitions the developing cell or sporangium into two unequal cellular compartments, the forespore and the mother cell. The σ^F factor is present before the formation of the polar septum and is activated in the forespore after septation. An important challenge is to understand the events that lead to the activation of σ^F in one cellular compartment. The activity of σ^F is governed by three regulatory proteins: SpoIIE, SpoIIAA, and SpoIIAB. Epistasis experiments indicate that these proteins constitute a regulatory hierarchy in which SpoIIE is an activator of SpoIIAA, SpoIIAA is an inhibitor of SpoIIAB, and SpoIIAB is an inhibitor of σ^F (*2*). Consistent with these relations, biochemical evidence indicates that SpoIIAB is an anti-sigma factor that binds to σ^F and holds it in an inactive complex and that SpoIIAA is an anti–anti-sigma factor that binds to SpoIIAB, thereby preventing it from binding to σ^F (*3*–*6*). SpoIIE is inferred to be an integral membrane protein with 8 to 10 membrane-spanning segments in the NH_2-terminal domain (residues 1 to 322) and with a large cytoplasmic domain in the COOH-terminus (residues 323 to 827) (*7*). SpoIIE becomes localized during division to the polar septum, which separates the forespore from the mother cell (*8*). However, the function of SpoIIE has been unknown (*2*).

SpoIIAA and SpoIIAB govern the activity of σ^F by a partner-switching mechanism in which the SpoIIAB·σ^F complex exchanges σ^F for SpoIIAA to release free and active σ^F (*4*, *5*, *9*). Partner switching is strongly influenced by the adenosine nucleotides adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in vitro. Thus, ADP stimulates the formation of the SpoIIAB·SpoIIAA complex and favors the release of σ^F. Conversely, ATP stimulates the formation of the SpoIIAB·σ^F complex and thus causes inhibition of σ^F (*4*, *5*). In addition, SpoIIAB is a protein kinase that uses ATP to phosphorylate SpoIIAA on Ser^{58} (*6*, *10*), which inactivates SpoIIAA and prevents it from binding to SpoIIAB (*4*, *5*).

To investigate the function of SpoIIE, a fusion protein consisting of the 505-residue COOH-terminal domain (residues 323 to 827) of SpoIIE joined to six histidine residues (hereafter called $SpoIIE_{323\text{-}827}$) was purified from *Escherichia coli* by nickel affinity chromatography (*11*) and incubated with SpoIIAA-P (where *P* indicates phospho-SpoIIAA) that was labeled with [^{35}S]methionine (Fig. 1A). Analysis of the products of the reaction by isoelectric focusing demonstrated that increasing concentrations of $SpoIIE_{323\text{-}827}$ converted ^{35}S-labeled SpoIIAA-P (whose isoelectric point was identical to that of SpoIIAA-P which had been labeled with ^{32}P) to a form whose isoelectric point was identical to that of unphosphorylated SpoIIAA (Fig. 1A, lanes 1 to 8). $SpoIIE_{323\text{-}827}$ also caused the loss of radioactivity from SpoIIAA-P that was labeled with ^{32}P, which confirmed that $SpoIIE_{323\text{-}827}$ is a serine phosphatase that had removed the phosphate residue from SpoIIAA-P (Fig. 1C) (*12*). The serine phosphatase activity of SpoIIE was greatly stimulated by Mn^{2+} (Fig. 1C), a cofactor for certain serine-threonine phosphatases (*13*). As a control for specificity, $SpoIIE_{323\text{-}827}$ failed to dephosphorylate the phosphorylated form of RsbV, a homolog of SpoIIAA that controls the activity of the *B. subtilis* stress response sigma factor σ^B (Fig. 1B) (*14*, *15*).

An additional control that provided physiological evidence for the function of the phosphatase activity came from the use of a mutant form of SpoIIAA in which Ser^{58} (the site of phosphorylation) was replaced with Thr (S58T). Cells producing the SpoIIAA-S58T mutant protein are blocked in the induction of σ^F-directed gene transcription (*9*). When incubated with SpoIIAB and ATP, the mutant protein readily underwent phosphorylation (*16*) (compare lanes 9 and 11 in Fig. 1A). However, incubation of the phosphorylated mutant protein with $SpoIIE_{323\text{-}827}$ failed to convert it back to the dephosphorylated form (Fig. 1A, lanes 11 and 12). Evidently, SpoIIAA-S58T is a substrate for the kinase but not for the phosphatase. This finding

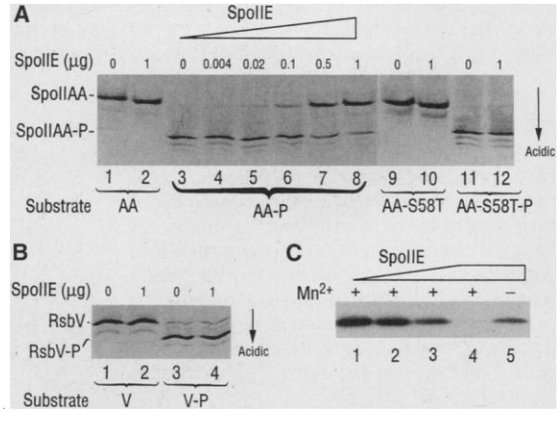

Fig. 1. The cytoplasmic domain of SpoIIE is a serine phosphatase. (**A** and **B**) Autoradiographs of the products of reactions containing phosphorylated or unphosphorylated SpoIIAA, SpoIIAA-S58T, or RsbV incubated with $SpoIIE_{323\text{-}827}$ and separated by isoelectric focusing. SpoIIAA, SpoIIAA-S58T, and RsbV were labeled with [^{35}S]methionine and phosphorylated as described (*22*). Next, the phosphorylated or unphosphorylated proteins were incubated for 30 min at 30°C with or without $SpoIIE_{323\text{-}827}$ in 40-μl reactions containing 2 mM $MnCl_2$. The reactions were then stopped by the addition of an equal volume of loading solution (*23*). The products of the reaction were separated on a 5% polyacrylamide isoelectric focusing slab gel (*23*). (**C**) Autoradiograph of the products of dephosphophorylation reactions containing ^{32}P-labeled SpoIIAA-P and various quantities of $SpoIIE_{323\text{-}827}$. ^{32}P-labeled SpoIIAA-P (*24*) was incubated in 50 μl of buffer A (*24*) in the presence or absence of 2 mM $MnCl_2$ (as indicated) and the following quantities of $SpoIIE_{323\text{-}827}$ for 30 min at 30°C: lane 1, 0 μg; lane 2, 0.2 μg; lane 3, 1 μg; and lanes 4 and 5, 5 μg. Reactions were terminated by the addition of SDS-polyacrylamide gel sample buffer. The reaction products were separated in an 18% SDS-polyacrylamide slab gel, which was fixed and dried.

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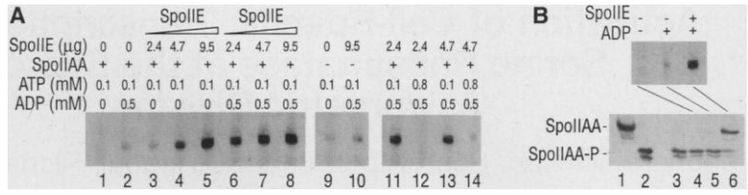

Fig. 2. SpoIIE$_{323-827}$ overcomes SpoIIAB-mediated inhibition of σ^F. (**A**) Autoradiographs of the products of run-off transcription reactions that had been subjected to electrophoresis in urea–8% polyacrylamide sequencing gels. Reaction mixtures (*25*) containing σ^F, SpoIIAB, 100 μM ATP, template DNA, and SpoIIAA (where indicated) were incubated for 10 min at 37°C. Next, aliquots (100 μl) were removed and supplemented with ADP, additional ATP, and SpoIIE$_{323-827}$ as indicated. Finally, transcription was carried out by the addition of core RNA polymerase (*21*). (**B**) The phosphorylation state of SpoIIAA correlates with the activity of σ^F. The top shows the products of σ^F-directed transcription, and the bottom shows the phosphorylation state of SpoIIAA from the transcription reaction mixtures. A reaction mixture containing σ^F, SpoIIAB, SpoIIAA, 100 μM ATP, and template DNA was incubated for 10 min at 37°C (*26*). Next, an aliquot (100 μl) was removed for isoelectric focusing (lane 3). Additional aliquots (200 μl) were removed and mixed with either nδ further components (lane 4), with 500 μM ADP (finalconcentration) (lane 5), or with 500 μM ADP (final concentration) and 38 μgof SpoIIE$_{323-827}$ (lane 6). After 5 min of incubation at 37°C, half (100 μl) of each of these reaction mixtures was removed and samples (containing about 260 ng of SpoIIAA) were subjected to isoelectric focusing (*23*) and protein immunoblot analysis with SpoIIAA antibodies (*9*). Transcription was then carried out in the remainder of each reaction mixture by the addition of core RNA polymerase (*21*). As isoelectric focusing markers, 380 ng of SpoIIAA (lane 1) and 380 ng of SpoIIAA-P (lane 2) were subjected to isoelectric focusing and protein immunoblot analysis.

explains the phenotype of cells that produce the mutant protein: SpoIIAA-S58T-P cannot undergo dephosphorylation in vivo and hence cannot overcome SpoIIAB-mediated inhibition of σ^F.

Next, we investigated whether SpoIIE$_{323-827}$ could reverse the SpoIIAB-mediated inhibition of σ^F-directed transcription. SpoIIAA, SpoIIAB, σ^F, and template DNA were incubated with 100 μM ATP under conditions that inactivate SpoIIAA by SpoIIAB-mediated phosphorylation and allow the formation of SpoIIAB·σ^F complexes (*9*). Transcription was initiated by the addition of core RNA polymerase in the presence or absence of SpoIIE$_{323-827}$. SpoIIE$_{323-827}$ effectively restored σ^F-directed RNA synthesis in the presence of SpoIIAB (Fig. 2A, lanes 1 through 8), and this restoration depended on the presence of SpoIIAA (Fig. 2A, compare lanes 8 and 10). Moreover, consistent with previous findings that ADP stimulates the formation of SpoIIAB·SpoIIAA complexes, the effect of 2.4 μg of SpoIIE$_{323-827}$ was strongly enhanced by the presence of 500 μM ADP (compare lanes 3 and 6 of Fig. 2A). (However, at higher concentrations SpoIIE$_{323-827}$ effectively restored transcription in the absence of added ADP; for example, compare lanes 5 and 8 of Fig. 2A.) Conversely, increasing the concentration of ATP from 100 μM to 800 μM, even in the presence of 500 μM ADP and 2.4 or 4.7 μg of SpoIIE$_{323-827}$, substantially prevented the restoration of σ^F-directed transcription (Fig. 2A, lanes 11 through 14).

Finally, to confirm that the effect of SpoIIE$_{323-827}$ was exerted at the level of dephosphorylation of SpoIIAA-P, isoelectric focusing and protein immunoblot analysis were performed to determine the state of phosphorylation of SpoIIAA in transcription reaction mixtures. In the absence of SpoIIE$_{323-827}$, SpoIIAA was principally present in the phosphorylated state, and in the presence of SpoIIE$_{323-827}$ SpoIIAA was largely unphosphorylated (Fig. 2B). Thus, the transcriptional activity of σ^F was correlated with the phosphorylation state of SpoIIAA.

The COOH-terminal region of SpoIIE and its homolog in *Bacillus megaterium* are similar to the COOH-terminal region of the product (RsbU) (*17*) of a regulatory gene in the pathway governing the activation of a stress response transcription factor (σ^B) in *B. subtilis* and marginally similar to the COOH-terminal region of another compo-

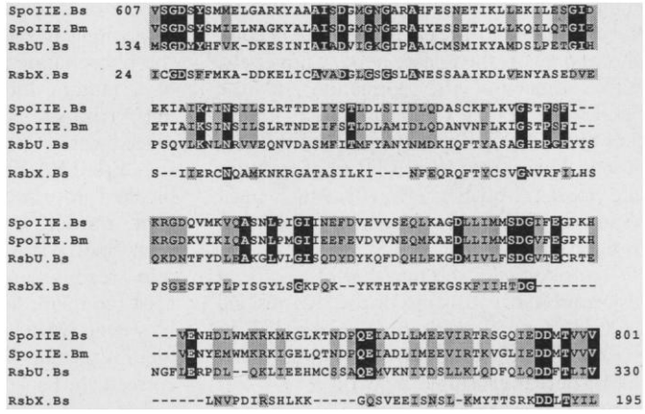

Fig. 3. A family of serine phosphatases. Shown is an alignment of the cytoplasmic domain of SpoIIE from *B. subtilis* (SpoIIE.Bs) (*20*) and from *B. megaterium* (SpoIIE.Bm; GenBank accession number U26836) with regions of RsbU (RsbU.Bs) (*17*) and RsbX (RsbX.Bs) (*14*) from *B. subtilis*. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Conservative amino acid substitutions between SpoIIE.Bs, SpoIIE.Bm, and RsbU.Bs are highlighted in gray, and identities are in black (*27*). Because of its weaker similarity, RsbX.Bs is presented separately. Amino acid sequence similarity was also noted (not shown) to a protein (IcfG; protein identification resource accession number S38573) of unknown function from *Synechocystis*. The numbers indicate the positions of the residues in each of the proteins; in the case of SpoIIE.Bm, the full predicted sequence is not available and hence the numbers are not indicated.

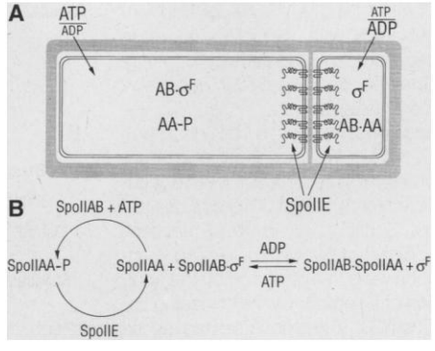

Fig. 4. A model for the establishment of cell-specific gene transcription. (**A**) The COOH-terminal (serine phosphatase) domain (represented by the curlicues) of SpoIIE could be more concentrated in the forespore (the small cell) than in the mother cell by virtue of being displayed on the cytoplasmic side of the membranes on either side of the division septum. The NH_2-terminal domain is represented by the series of transmembrane segments. The stippled material represents the cell wall, and the parallel double lines represent the cytoplasmic membranes. (**B**) The pathway extending from SpoIIE to the activation of σ^F. The phosphorylation state of SpoIIAA is governed by a cycle of opposing kinase (SpoIIAB) and phosphatase (SpoIIE) activities. Dephosphorylated SpoIIAA displaces σ^F from the SpoIIAB·σ^F complex to yield SpoIIAB·SpoIIAA and free and active σ^F. ADP and ATP oppositely influence partner switching and hence indirectly govern phosphorylation by determining whether the SpoIIAB kinase is sequestered in an inactive complex with SpoIIAA. Activation of σ^F could be augmented by a selective decrease in the ratio of ATP to ADP in the forespore as depicted in (A) or by an overall drop in the ratio of ATP to ADP in the sporangium.

nent (RsbX) (*14*) of the σ^B regulatory system (Fig. 3). The activity of σ^B is controlled by homologs of SpoIIAA and SpoIIAB called RsbV and RsbW, respectively (*14*, *15*). RsbV is an anti–anti-sigma factor that binds to RsbW, and RsbW is an anti-sigma factor of σ^B and also a protein kinase that phosphorylates RsbV (*15*). RsbU and RsbX may be serine phosphatases that dephosphorylate RsbV-P or the phosphorylated form of an additional related protein in the σ^B system encoded by *orfS* (*17*).

Combined with other results (3–6, 8), the discovery that the COOH-terminal region of SpoIIE is a serine phosphatase allows the assignment of a function for each step in the pathway that extends from asymmetric division to cell-specific activation of σ^F. During the partitioning of the sporangium into two cellular compartments, SpoIIE localizes to the polar septum (Fig. 4A), where its COOH-terminal cytoplasmic domain activates SpoIIAA-P by dephosphorylation (Fig. 4B). Next, dephosphorylated SpoIIAA interacts with the SpoIIAB·σ^F complex, which causes an exchange of partners that results in the formation of the SpoIIAB·SpoIIAA complex and the release of free and active σ^F (Fig. 4B). If the cytoplasmic domain of SpoIIE is displayed equally on the forespore and mother-cell sides of the polar septum, then the phosphatase would be more concentrated in the forespore than in the mother cell because of the smaller volume (no more than one-fifth as large) of the former (Fig. 4A). This higher effective concentration of phosphatase would result in more σ^F activity in the forespore than in the mother cell; however, this may be insufficient to account for the strict cell-specific activity of σ^F (*18*). In confirmation and extension of previous results showing that partner switching by, and the kinase activity of, SpoIIAB is strongly influenced by ADP and ATP (*4*, *5*), the capacity of the SpoIIE phosphatase to counteract the effect of the SpoIIAB kinase was partially dependent on ADP and strongly inhibited by ATP. Thus, the cell-specific activation of σ^F could be a composite consequence of the septal location of SpoIIE and an alteration in cellular adenosine nucleotide levels (Fig. 4A) (*19*).

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11. A Bam HI site was generated 1515 bp upstream of the *spoIIE* stop codon by polymerase chain reaction–mediated DNA amplification from a cloned copy of *spoIIE*. Next, a Bam HI–Dra I DNA fragment containing 505 codons from the 3′ end of the *spoIIE* coding sequence was subcloned into pRSETB (Invitrogen), thereby adding six histidine codons to the 5′ end of the truncated gene. The modified truncated gene was expressed in *E. coli* strain BL21(DE3) (Novagen) by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Inclusion bodies were solubilized in 8 M urea, and the protein was applied to a Ni-NTA agarose column (Qiagen) and refolded on the column with a decreasing gradient of urea. The protein was then eluted with imidazole, and desalted into 100 mM tris-HCl (pH 8), 200 mM NaCl, and 2 mM dithiothreitol (DTT). Next, it was concentrated in a Centricon-10 (Amicon), diluted with 1 volume of 100% glycerol, and stored at −20°C.
12. Silver staining confirmed that the loss of radioactivity from ^{32}P-labeled SpoIIAA-P was not due to SpoIIE$_{323-827}$-induced degradation of SpoIIAA. Substantial dephosphorylation required approximately stoichiometric amounts of SpoIIE$_{323-827}$ and SpoIIAA, perhaps indicating that SpoIIE$_{323-827}$ was inefficiently renatured in our protocol (*11*) or that the NH_2-terminal, integral membrane portion of the protein is needed for full activity. Alternatively, SpoIIE may normally be present and act in roughly equimolar concentration to that of SpoIIAA during sporulation. Strictly speaking, these results do not distinguish between SpoIIE being the phosphatase or an activator of a SpoIIAA autophosphatase. For simplicity, the former is assumed to be the case. Consistent with the idea that SpoIIE is the phosphatase, SpoIIAA-P was highly stable and little or no dephosphorylation was observed in the absence of SpoIIE$_{323-827}$. The phosphate moiety of SpoIIAA-P is evidently liberated by hydrolysis because little or no ^{32}P was transferred to an acid-stable (hydroxyl) moiety on SpoIIE$_{323-827}$.
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16. Phosphorylation presumably occurred at Thr^{58} because an Ala^{58} substitution mutant does not undergo phosphorylation (*5*), but conceivably the S58T mutant undergoes phosphorylation at a serine residue in the vicinity of amino acid 58.
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19. Alternatively, cell-specific activation of σ^F could be governed exclusively by the SpoIIE phosphatase if, by an unknown mechanism, the phosphatase activity is restricted to the forespore face of the septum.
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21. Transcription buffer contained 40 mM tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.4 mM DTT, 200 μM guanosine triphosphate, 200 μM uridine triphosphate, 0.02 μM unlabeled cytosine triphosphate (CTP), and 0.1 μCi/μl of [α-^{32}P]CTP (10 μCi/μl, 3.3 μM; NEN). Transcription reactions were initiated by the addition of 200 ng of *B. subtilis* core RNA polymerase (*3*). Purification of σ^F was as described (*9*). The template for transcription was pLD14 that had been linearized with Hinc II, which yielded run-off transcripts of 87 nucleotides (*9*). pLD14 contains a strong σ^F-dependent promoter [*sspE-2G*; D. Sun *et al.*, *J. Bacteriol.* **173**, 7867 (1991)] fused upstream of DNA lacking adenosine nucleotides on the nontemplate strand (*9*). Transcription reactions were terminated after 15 min at 37°C by the addition of heparin (500 μg/ml, final concentration) and unlabeled CTP (200 μM, final concentration). After incubation for 5 min at 37°C, RNA in the reaction mixtures was precipitated twice with 3 volumes of ethanol/3 M sodium acetate [40:1 (v/v)] in the presence of 13.5 μg of carrier yeast RNA, dried, and resuspended in 10 μl of formamide loading solution. Samples were boiled and loaded on urea–8% polyacrylamide sequencing gels.
22. *spoIIAA*, *spoIIAA-S58T*, and *rsbV* were expressed in *E. coli* with an IPTG-inducible T7 RNA polymerase system, and the protein products were labeled with [^{35}S]methionine as described (*4*). The *spoIIAA* expression plasmid and strain have been described (*4*). The construction of the *spoIIAA-S58T* expression plasmid (containing a mutant *spoIIAA* in which Ser codon 58 was converted to a Thr codon) and the construction of the *rsbV* T7-RNA polymerase expression plasmid and strain were as described (*9*). To generate ^{35}S-labeled SpoIIAA-P and ^{35}S-labeled SpoIIAA-S58T-P, cell pellets of the expression strains corresponding to 2 ml of [^{35}S]methionine–labeled cells were resuspended in 1 ml of lysis buffer (*9*) and the cells lysed by freeze-thawing. Insoluble debris was removed by centrifugation for 10 min at 4°C. ATP was added to a final concentration of 1 mM, and 200 μl of the extract was passaged five times over a 20-μl column containing 4 μg of purified SpoIIAB immobilized on a solid support (Affi-Gel-10; Bio-Rad) (*9*). This procedure generates quantitatively phosphorylated ^{35}S-labeled SpoIIAA and SpoIIAA-S58T free of SpoIIAB. This ^{35}S-labeled SpoIIAA-P and SpoIIAA-S58T-P (20 μl each) was used in each dephosphorylation experiment in Fig. 1A. ^{35}S-labeled RsbV-P was generated in a similar manner with the use of purified RsbW, except that the ^{35}S-labeled RsbV was passaged five times over a 20-μl column containing approximately 4 μg of RsbW immobilized on Affi-Gel-10 (*9*). As verification of phosphorylation, ^{35}S-labeled SpoIIAA-P and ^{35}S-labeled RsbV-P had isoelectric points identical to those of the corresponding ^{32}P-labeled proteins.
23. The loading solution for isoelectric focusing contained 8 M urea, 12% ampholytes (pH 3 to 10) (Pharmalyte; Pharmacia), 2% Triton X-100, 1% 2-mercaptoethanol, and 0.1% bromophenol blue. The 5% polyacrylamide isoelectric focusing slab gels contained 2.5% (w/v) ampholytes (pH 3 to 10) (Pharmalyte; Pharmacia) and 8 M urea and were run for 2 hours at 300 V. The anolyte was 10 mM phosphoric acid, and the catholyte was 20 mM NaOH. The gels of Fig. 1, A and B, were fixed for 10 min in 10% trichloroacetic acid, rinsed in water, and dried. The weaker radioactive species are isoelectric variants of SpoIIAA and RsbV that presumably arose by deamination or other protein modification during isolation. The gel of the bottom of Fig. 2B was soaked in transfer buffer (48 mM tris base, 39 mM glycine, 0.037% SDS, and 20% methanol) for 30 min before transfer to Immobilon-P membrane (Millipore) and subjected to protein immunoblotting (*9*).
24. Purification of SpoIIAA and SpoIIAB was as described (*9*). Purified SpoIIAA (6 μg) was phosphorylated in 100 μl of reaction mixture containing 20 μg of SpoIIAB immobilized on Affi-Gel-10 (Bio-Rad) (*9*) and 10 μCi of [γ-^{32}P]ATP (New England Nuclear) in buffer A [20 mM Hepes (pH 7.5), 100 mM NaCl, 10% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP] for 30 min at room temperature. SpoIIAB was removed by centrifugation, and 5-μl aliquots of SpoIIAA-^{32}P (corresponding to approximately 300 ng of protein) were used in the dephosphorylation experiments of Fig. 1C.
25. Reaction mixtures contained 3 μg of σ^F, 4 μg of SpoIIAB, 17.6 μg of SpoIIAA, 100 μM ATP, and 20 μg of template in 1 ml of transcription buffer (*21*) (lanes 1 to 8); or 1.2 μg of σ^F, 1.6 μg of SpoIIAB, 100 μM ATP, and 8 μg of template in 400 μl of buffer (lanes 9 and 10); or 3 μg of σ^F, 4 μg of SpoIIAB, 17.6 μg of SpoIIAA, 100 μM ATP, and 20 μg of template in 1 ml of buffer (lanes 11 to 14).
26. The reaction mixture contained 2.4 μg of σ^F, 3.2 μg

of SpoIIAB, 14.1 μg of SpoIIAA, 100 μM ATP, and 16 μg of template in transcription buffer (800 μl) (*21*).
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Central Command Neurons of the Sympathetic Nervous System: Basis of the Fight-or-Flight Response

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During stress, the activity of the sympathetic nervous system is changed in a global fashion, leading to an increase in cardiovascular function and a release of adrenal catecholamines. This response is thought to be regulated by a common set of brain neurons that provide a dual input to the sympathetic preganglionic neurons regulating cardiac and adrenal medullary functions. By using a double-virus transneuronal labeling technique, the existence of such a set of central autonomic neurons in the hypothalamus and brainstem was demonstrated. These neurons innervate both of the sympathetic outflow systems and likely function in circumstances where parallel sympathetic processing occurs, such as in the fight-or-flight response.

The sympathetic nervous system regulates a broad range of visceral functions and, during extreme emotional or physical states, activates both the cardiovascular and adrenal catecholamine systems for homeostatic adjustments (*1*). The central nervous system (CNS) neurons responsible for coactivation of these autonomic changes are thought to be governed by a common set of central command neurons that provides dual projections to the sympathetic outflow systems that control the heart and adrenal gland. Although this biological idea was described in the late 1920s (*1*) and is taught as a basic principle of autonomic function, it has not been possible to define the command neurons and CNS circuits responsible for this response, because of the technical limitations. We have now developed a double-virus transneuronal labeling method to localize and to chemically characterize the central command neurons.

The general scheme of this study is presented in Fig. 1A. Two different genetically engineered forms of the Bartha strain of pseudorabies virus (PRV) were used as transneuronal tracers (*2*); each expressed a unique marker antigen in infected host cells (Fig. 1B). Both produce specific infections within functionally related chains of neurons. One virus was injected into the stellate ganglion—the major sympathetic ganglion that innervates the heart (*3*)—and the other virus was injected into the ipsilateral adrenal gland of anesthetized Sprague-Dawley rats or vice versa (*4*). After 4 days, rats were anesthetized and perfused with fixative, and their brains processed by a triple-antibody immunohistochemical procedure for the two unique virally induced cellular markers (gC viral glycoprotein and β-galactosidase) and also stained for a neurotransmitter enzyme or neurotransmitter (choline acetyltransferase, phenylethanolamine-*N*-methyltransferase, tyrosine hydroxylase, serotonin, or oxytocin) (*5*). A total of 20 rats contained double-virus infections; eight of these had CNS patterns of infection for both viruses that were similar to those found in earlier studies in which a single strain of PRV was injected into the adrenal gland (*6*) or the stellate ganglion (*7*).

The brain sites that were transneuronally labeled with the two different viruses that had been injected into the terminal fields of the sympathoadrenal and stellate sympathetic preganglionic neurons are illustrated in Fig. 2. Three areas of the medulla oblongata were labeled: (i) rostral ventrolateral medulla; (ii) rostral ventromedial medulla, which includes the lateral paragigantocellular reticular nucleus, parapyramidal nucleus, and ventral and pars alpha regions of the gigantocellular reticular nuclei; and (iii) caudal raphe nuclei (raphe magnus, raphe pallidus, and raphe obscurus). Monoaminergic medullary neurons contribute to this projection. C1 adrenergic neurons (Fig. 3), in both the rostral

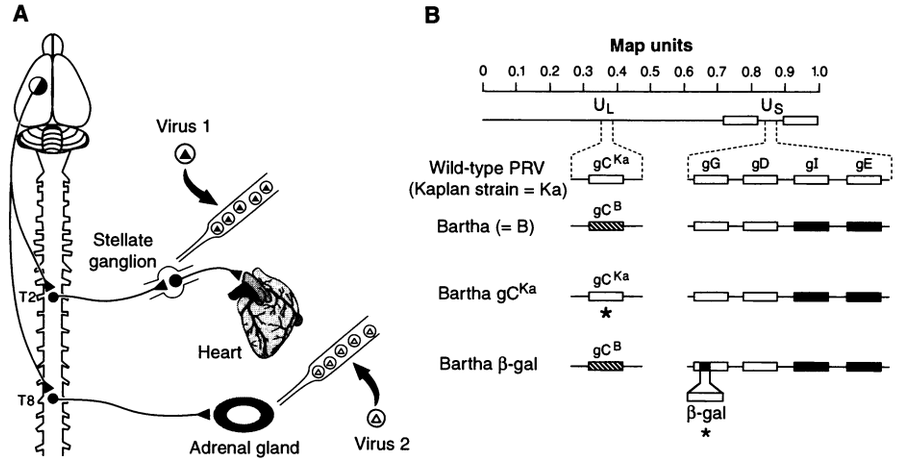

Fig. 1. (**A**) The CNS sites that regulate the sympathetic outflow of both the stellate ganglion and adrenal gland were identified by a double-virus transneuronal labeling method. In the same animal, one virus was injected into the stellate ganglion and the second virus into the ipsilateral adrenal gland. Each virus produced a unique intracellular marker in infected host neurons, and some neurons contained both markers, indicating that they regulate both sympathetic systems. (**B**) Two genetically modified forms of Bartha PRV used for transneuronal labeling of central sympathetic circuits (Bartha-gC^{Ka} PRV and Bartha β-galactosidase PRV). The genomes of these two viruses differ from the wild-type PRV and the original attenuated Bartha strain. Each modified virus contained a gene that produced a different intracellular antigen in the infected host neurons that could be detected by specific antibodies (asterisks). Bartha-gC^{Ka} PRV produced the wild-type form of the gC glycoprotein, which was detected by a mouse monoclonal antibody. Bartha β-galactosidase PRV was detected by a goat polyclonal antibody directed against β-galactosidase (*2*). U_L, unique long segment; U_S, unique short segment. Black boxes, deleted sequences; striped boxes, altered sequences.

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