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The Phosphorelay Signal Transduction Pathway in the Initiation of *Bacillus subtilis* Sporulation

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Abstract The formation of spores in *Bacillus subtilis* is a developmental process under genetic control. The decision to either divide or sporulate is regulated by the state of phosphorylation of the Spo0A transcription factor. Phosphorylated Spo0A (Spo0A ~ P) is both a repressor and an activator of transcription depending on the promoter it is affecting. Spo0A ~ P is the end product of the phosphorelay, a signal transduction system linking environmental information to the activation of sporulation. Activation or deinhibition of two ATP-dependent kinases, KinA and KinB, to phosphorylate the Spo0F secondary messenger initiates the phosphorelay. Spo0F ~ P is the substrate for the Spo0B protein, a phosphoprotein phosphotransferase which transfers the phosphate group to Spo0A. The Spo0A ~ P formed from this pathway orchestrates transcription events during the initial stage of spore development through direct effects on a variety of promoters and through the use of other transcription factors, termed transition state regulators, whose activity it controls. Because commitment to sporulation has serious cellular programming consequences and is not undertaken capriciously, the phosphorelay is subject to a variety of complex controls on the flow of phosphate through its components.

Key words: transcription, phosphorylation, kinases, 0A box, phosphategroup

Sporulation is one mechanism that microorganisms have developed for survival under adverse conditions. Studies of the genetics of this developmental process have been carried out over the last 30 years in *Bacillus subtilis* and some aspects of the regulation of sporulation are now understood. The initiation of sporulation involves a serious commitment on the part of the cell to shut down division and activate a large number of morphogenetic processes; therefore, it should not be surprising that a complicated regulatory system would evolve to control entry into sporulation. The concept has evolved that the cell monitors its environment and metabolic potential during a window in the division cycle and, from this information, makes a decision to either continue DNA synthesis and division or initiate sporulation (Fig. 1). The nature of the information, the mechanism by which the cell monitors this information, and the pathway of signal transduction to activate transcription remained a mystery until recently. One successful approach to solving this problem was through the use of mutants deficient in the monitoring and signal transduction systems. Mutants were obtained, *spo0* mutants, that blocked the very earliest steps in sporulation [Hoch, 1976]. These mutants appeared to be "locked" in log phase and unable to recognize any signal to initiate sporulation-specific transcription. *Spo0* mutations mapped in six genetically distinguishable loci, *spo0A, spo0B, spo0E, spo0F, spo0K,* and *spo0H* that ultimately were found to form the core of the signal transduction mechanism for the initiation of sporulation.

THE PHOSPHORELAY

The key to understanding transcription regulation at the initiation of sporulation is understanding the control of activation of the Spo0A transcription factor. Spo0A is a typical response regulator protein of the sensor-response regulator two-component pair class [Stock et al., 1990]. Genetic evidence implicated it in transcriptional regulation and its primary sequence showed its relationship to regulators of sensor-regulator pairs [Trach et al., 1985]. It was shown that certain mutational alterations in the Spo0A protein could suppress the need for the *spo0B, spo0E, spo0F,* and *spo0K* gene products, suggesting that these latter genes code for proteins

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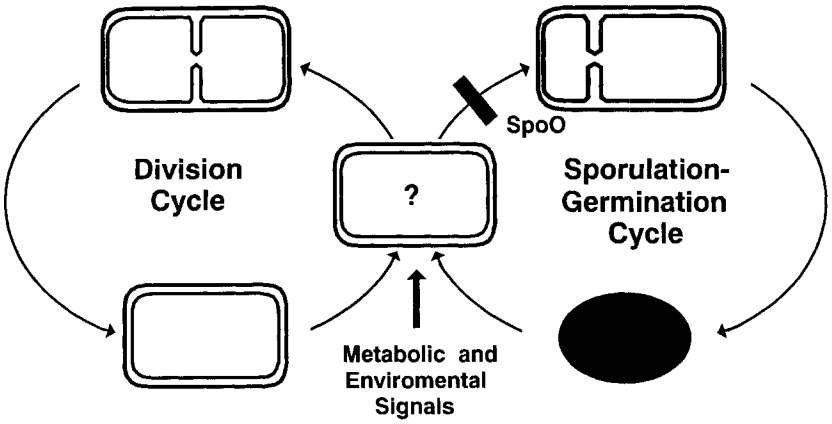

Fig. 1. Division and sporulation cycles in *Bacillus subtilis*. A cell deciding which cycle to enter depends upon metabolic and environmental signals to determine its developmental fate. These signals are processed through the phosphorelay.

that activate Spo0A [Hoch et al., 1985]. When it was discovered that activation of response regulators occurred by phosphorylation via the sensor component [Ninta and Magasanik, 1986], this implicated a component of the sensor-kinase class in Spo0A activation. The problem was that the deduced gene products of the *spo0B, spo0E, spo0F,* and *spo0K* genes were clearly not related in primary structure to sensor kinases. Meanwhile, the *spo0H* gene had been shown to code for a sigma factor subunit of RNA polymerase [Dubnau et al., 1988]. To further complicate matters, the deduced sequence of the *spo0F* gene product was homologous to response regulators [Trach et al., 1985]. Thus, the genetic studies identified two response-regulators, several genes of unknown function and no sensor-kinases.

The way out of this dilemma was provided by the discovery of a sensor-kinase as the product of a putative stage II sporulation locus, *spoIIJ* [Antoniewski et al., 1990]. The product of this locus, KinA, was found to be capable of phosphorylating both Spo0F and Spo0A proteins but was much more active on Spo0F [Perego et al., 1989]. What was still unclear was the identity of the kinase directly responsible for Spo0A phosphorylation and what role phosphorylated Spo0F played in the initiation of sporulation.

Purification of the *spo0* gene products in quantity from overexpressing strains allowed a biochemical solution to this genetic problem. The product of the *spo0B* locus when added in vitro to reaction mixtures containing ATP, KinA, Spo0F, and Spo0A proteins was found to catalyze the transfer of phosphate from Spo0F ~ P to Spo0A, resulting in Spo0A ~ P [Burbulys et al., 1991]. Thus, Spo0A is not directly phosphorylated by an ATP-dependent kinase but rather is a substrate for the Spo0B phosphoprotein phosphotransferase which catalyses the transfer of phosphate from one response regulator to another. The sequential transfer of phosphate from ATP to KinA to Spo0F to Spo0B and finally to Spo0A was termed a phosphorelay (Fig. 2).

Several features of the phosphorelay are unique among signal transduction systems using sensor-response regulator pairs. None of the other systems has a counterpart to the Spo0B phosphoprotein phosphotransferase. Although Spo0F superficially resembles CheY in size and structure, unlike CheY it appears not to have a regulatory role, but instead serves as a secondary messenger for the accumulation of phosphate groups from at least two kinases with different environmental stimuli. This allows the additive effects of several kinases and, therefore, various environmental conditions to regulate the initiation of sporulation through a "cumulative environsensory mechanism" [Trach et al., 1990].

REGULATION OF PHOSPHATE TRANSFER IN THE PHOSPHORELAY

Since the flow of phosphate through the phosphorelay is the major factor determining the cellular level of Spo0A ~ P this flow is controlled at multiple points. There are two kinases, KinA and KinB, that act on Spo0F. KinA is a soluble cytoplasmic enzyme. Its activity is inhibited by *cis*-unsaturated fatty acids [Strauch

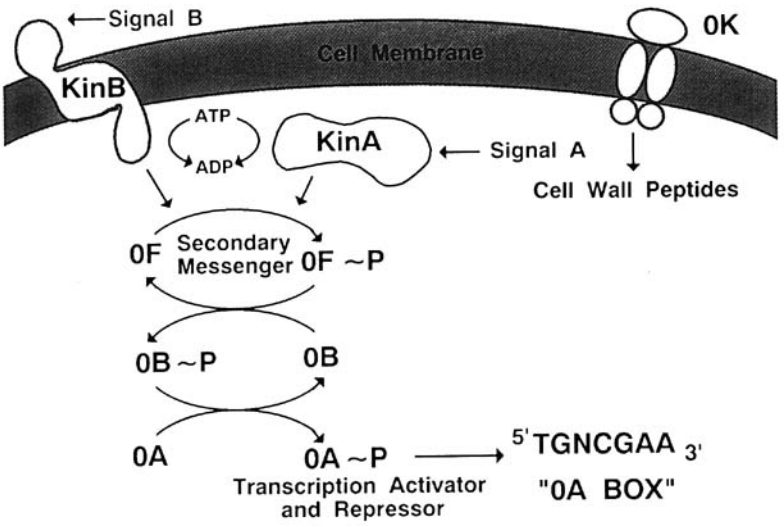

Fig. 2. The phosphorelay signal transduction system. Signals entering through KinA or KinB are processed to activate the Spo0A transcription factor by phosphorylation. 0K is the oligopeptide permease system.

et al., 1991] in a manner analogous to eucaryotic protein kinase C [Bell and Burns, 1991]. The regulatory basis for this inhibition is unclear. Fatty acids might act as a secondary messenger transmitting environmental or metabolic information. In *B. subtilis cis*-unsaturated fatty acids are a very minor portion of the total fatty acids, suggesting that fatty acids of this type may play special roles such as being associated with specific structures or enzyme complexes with an essential spacial distribution. One example of such a specific structure that comes to mind is the septation apparatus. The role of fatty acid inhibition is simply not understood, even in the case of protein kinase C, and it has not been proven that such fatty acids are inhibitory in vivo. No effectors have been found that activate KinA.

Mutants deficient in KinA still sporulate, although the rate of sporulation is slower. This reflects the activity of a second pathway using another kinase, KinB [Thach and Hoch, 1993]. KinB is likely to be located in the cytoplasmic membrane since the deduced primary structure of the enzyme has six probable membrane spanning regions with only a bare minimum of the molecule exposed on the outer surface (K. Trach and J. Hoch, unpublished data). This differs from most membrane bound sensor kinases that have substantial portions of the molecule in the periplasmic space or on the membrane surface exposed to the environment, probably as a ligand binding domain [Stock et al., 1990]. Whether the membrane location of KinB indicates an environmental stimulus for enzymatic activity or some other role requiring a membrane location, the effector molecule stimulating or inhibiting KinB has not been identified. Double mutants, *kinA kinB,* are severely deficient in sporulation suggesting that these two kinases are the major kinases feeding phosphate to Spo0F and the phosphorelay.

The kinetic parameters of kinase A have been extensively studied. KinA has a high affinity for Spo0F, $k_m \sim 1\ \mu M$, and it is activated by Spo0F. KinA has a labile phosphatase activity that may play a role in regulation of Spo0F levels. Kinetic studies of KinB have not been undertaken primarily because of its refractive membrane location. Spo0F is the presumed substrate for KinB, although this was deduced solely from genetic studies; *spo0F* mutants are completely sporulation deficient, negating the possibility that KinB acts on another response regulator that serves as a substrate for Spo0B. This result suggests further that no other unrelated response regulators are significant substrates for Spo0B. There is some level of crosstalk directly on Spo0F, however; enough Spo0A ~ P is formed in a *kinA kinB* double mutant to allow repression of the *abrB* gene, a sensitive indicator of in vivo Spo0A ~ P levels. This repression does not occur in a *spo0F* or *spo0B* mutant showing that there is little, if any, crosstalk directly on Spo0A.

Other than its role as a secondary messenger, Spo0F does not appear to have a crucial regula-

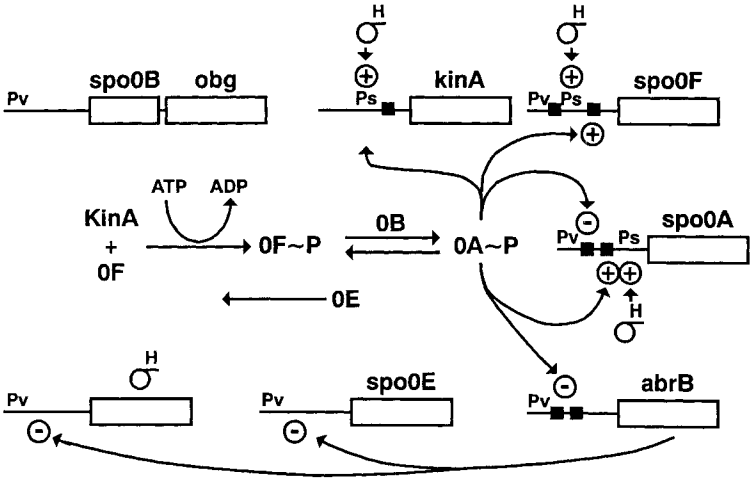

Fig. 3. Transcription relationships among component genes of the phosphorelay. Vegetative σ^A, P_V, and sporulation σ^H, P_S, promoters are indicated. Black boxes in the promoters are locations of 0A boxes, the site of Spo0A binding.

tory function. This is the interpretation of the observation that *spo0F* deletion mutants and *spo0B* mutants can be suppressed to wild-type levels of sporulation by *sof* mutations in Spo0A that may allow other, unrelated, kinases to phosphorylate it directly [Kawamura and Saito, 1983; Sharrock et al., 1984; Spiegelman et al., 1990].

Negative regulation of the phosphorelay occurs through the *spo0E* gene product. This gene was originally identified as a *spo0* mutant by a decrease in sporulation [Perego and Hoch, 1987]. Alleles of the locus were found to be nonsense mutations in the carboxyl half of a small protein of 9791 Da, which led to the assumption that loss of function of the Spo0E protein accounted for the sporulation phenotypes of such mutants. However, when a deletion mutant was constructed and shown to be sporulation proficient, this conclusion had to be reevaluated [Perego and Hoch, 1991]. Deletion mutants were found to segregate Spo$^-$ colonies with secondary mutations in or near the *spo0A, spo0B,* or *spo0F* genes suggesting that the deletion of *spo0E* results in an increased pressure to sporulate which leads to the accumulation of suppressors of this developmental drive through mutations in the phosphorelay. How the spo0E protein negatively controls the flux of phosphate through the phosphorelay is unclear. However, it is certain that deletion of the *spo0E* gene does not alter the transcriptional regulation of the *spo0A, spo0B, spo0F,* or *kinA* genes. Perhaps the Spo0E protein inhibits one of the steps of the pathway and, in the absence of this inhibition, Spo0A ~ P accumulates at inappropriate times.

TRANSCRIPTIONAL REGULATION OF THE PHOSPHORELAY

The level of Spo0A ~ P in the cell is the major determinant of the decision to sporulate. This level is regulated by controlling the flow of phosphate through the phosphorelay and by controlling transcription of key components of the phosphorelay. Figure 3 shows an overview of the transcription regulatory mechanisms acting on the phosphorelay. Paradoxically, the crucial factor controlling Spo0A ~ P levels is the level of Spo0A ~ P itself. Under conditions of nutrient excess exponential growth, the level of Spo0A ~ P in the cell is very low. This results from several factors including catabolite repression of *spo0A* transcription [Weickert and Chambliss, 1990], assumed lack of activating signals for the kinases, KinA and KinB, and repression of the sigma factor gene spo0H by the AbrB transition state regulator and other factors [Dubnau et al., 1987]. Both the *spo0A* and *spo0F* genes have dual promoters; a σ^A promoter and a σ^H promoter [Chibazakura et al., 1991]. A low level of both *spo0A* and *spo0F* transcription is maintained under nutrient excess conditions by initiation at the σ^A dependent promoters. As conditions became less favorable for growth, a low level of Spo0A ~ P accumulates in the cell. This level is sufficient to repress *abrB* which relieves repression of the *spo0H* and *spo0E* genes, among

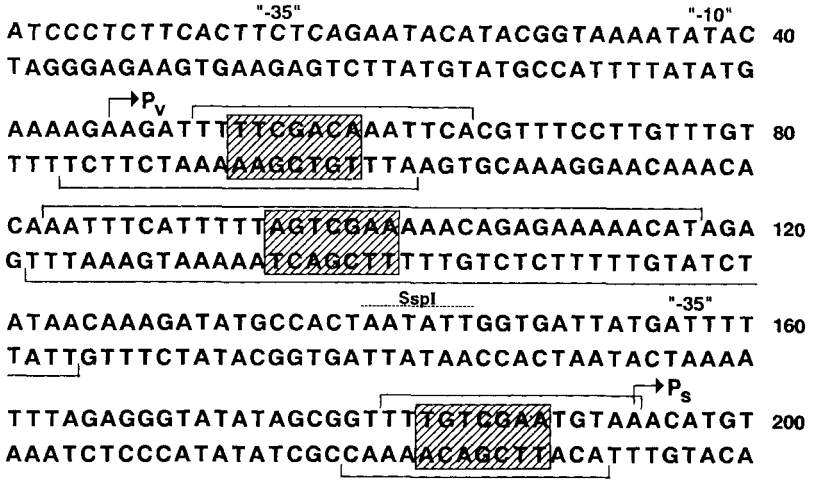

Fig. 4. Nucleotide sequence of the *spo0A* promoter. Shaded areas are the 0A boxes. Overlines and underlines show the extent of Spo0A binding to each strand in footprint analyses. P_V and P_S as in Figure 3.

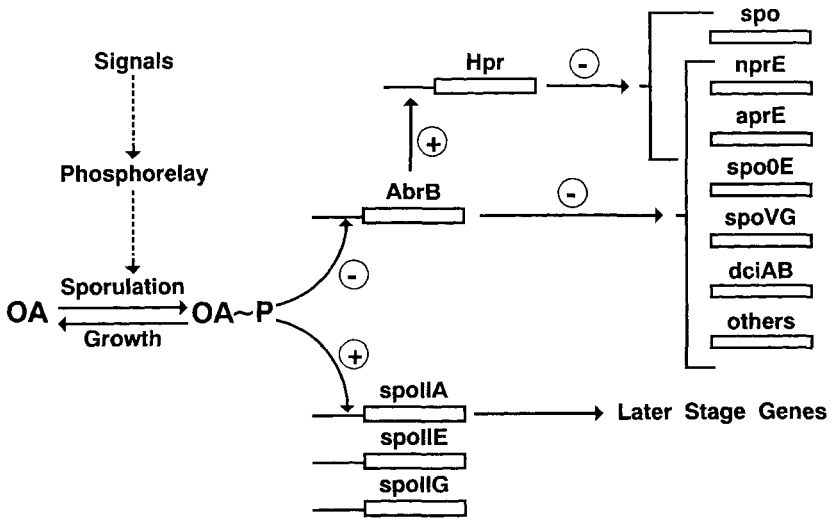

Fig. 5. General scheme for Spo0A ~ P control of transcription at the onset of sporulation.

others. High level transcription of *spo0A, spo0F,* and *kinA* is dependent on σ^H the product of the *spo0H* gene [Predich et al., 1992].

The *spo0A* promoter has several binding sites for Spo0A ~ P located between the σ^A and the σ^H promoters [Strauch et al., 1992a] (Fig. 4). As the level of Spo0A ~ P rises at the end of exponential growth, binding of Spo0A ~ P to these sites represses transcription from the σ^A promoter and activates transcription from the σ^H promoter [Chibazakura et al., 1991]. Presumably binding of Spo0A ~ P to the 0A box just downstream of the P_V (σ^A) promoter represses transcription from P_V and binding at this site as well as to the 0A box just downstream (nucleotides 83 to 117, Fig. 4) individually or in combination activates transcription from the P_S (σ^H) promoter. We believe that the 0A box coincident with the −10 region of P_S acts to repress transcription from P_S when the Spo0A ~ P concentration reaches a specific level. Similarly, the *spo0F* σ^H promoter is activated by Spo0A ~ P binding to an upstream site [Strauch et al., 1992b] and a second downstream site of Spo0A ~ P binding may modulate this transcription. The *kinA* gene is also transcribed from a σ^H promoter and has an integral downstream Spo0A ~ P binding site which may down regulate *kinA* transcription. This arrangement of the *spo0A* and *spo0F* promoters provides a positive feed-

back loop to increase transcription of the components of the phosphorelay in response to low levels of Spo0A ~ P [Strauch et al., 1992b]. In contrast, *spo0B* transcription does not seem to be controlled by Spo0A ~ P. Since Spo0B plays an enzymatic role in the phosphorelay its activity may not be strictly dependent on its concentration as is surely the case for Spo0A.

TRANSCRIPTIONAL ACTIVITY OF Spo0A ~ P

Spo0A regulates the initiation of sporulation by binding to a specific sequence, TGNCGAA, in the promoter regions of genes it controls [Strauch et al., 1990]. Both the phosphorylated and non-phosphorylated forms of Spo0A bind to this sequence and, at least in the case of the *abrB* gene, the phosphorylated form has an apparent high affinity of binding [Trach et al., 1991]. The *abrB* promoter is very sensitive to low levels of Spo0A ~ P, in vivo, and this may be a consequence of the tandem repeat of 0A boxes in this promoter separated by one helical turn of the DNA duplex. Such an arrangement may allow cooperativity between two molecules of Spo0A ~ P and stabilize the repressor-DNA complex. The phosphorylated form of Spo0A is the only active species of this protein. Mutants D10N and D56N of Spo0A which cannot be phosphorylated to Spo0A ~ P [Burbulys et al., 1991] are unable to repress the *abrB* promoter [Strauch et al., 1992a]. Purified preparations of such mutant proteins bind and footprint as well as unphosphorylated Spo0A to the *abrB* promoter, however [Strauch et al., 1992a]. It seems likely that the apparent higher affinity of Spo0A ~ P for this promoter may arise from a lower dissociation constant brought about by interaction of two Spo0A ~ P molecules.

Promoters that are activated by Spo0A ~ P show complex kinetics [Trach et al., 1991; York et al., 1992; Satola et al., 1992]. The SpoIIE promoter contains several 0A boxes that differ in their relative affinities for Spo0A [York et al., 1992]. Spo0A binding to the −35 region of the *spoIIE* promoter may facilitate interaction of σ^A with this promoter. The same is probably true for the SpoIIG promoter [Satola et al., 1992]. The large differences in apparent affinity between Spo0A and Spo0A ~ P are not observed with the SpoIIG promoter, and probably Spo0A ~ P serves to stabilize the ternary complex of DNA-Spo0A ~ P-RNA polymerase (G. Spiegelman, personal communication), perhaps through interactions with the C-terminal region of Spo0A [Perego et al., 1991]. It is clear both from mutant studies and in vitro transcription assays that only the phosphorylated form of Spo0A stimulates transcription from these promoters.

HOW SPORULATION IS CONTROLLED

It should be evident that the cellular level of phosphorylated Spo0A transcription factor is the crucial agent determining cellular fate, i.e., division or sporulation. Nature has provided an interesting mechanism to allow the cell to amplify the effects of Spo0A ~ P concentration through the use of transition state regulators. These regulators are generalized transcription repressors or perhaps better termed "preventers" that affect genes normally transcribed after the end of exponential growth in that transition period between growth and sporulation (Fig. 5). These factors are not necessarily the regulators of the genes in question but rather serve to prevent the expression of such genes during exponential growth by binding to their promoters. Some of the genes affected are required for sporulation and some are not. Probably the most important of the transition state regulators is the product of the *abrB* gene. As was previously discussed, *abrB* gene transcription is very sensitive to low levels of Spo0A ~ P. Under conditions conducive to growth where the cellular Spo0A ~ P level is low, the *abrB* gene is maximally expressed. The resulting high cellular level of AbrB prevents transcription of a large number of genes including proteases, some sporulation genes (e.g., *spo0E* and *spo0H*), antibiotic pathways, the entire competence pathway, and others. Furthermore, AbrB has positive effects on the transcription of another regulator, Hpr, which represses another set of genes. The *abrB* system guards against unnecessary and detrimental gene expression during exponential growth when all the available carbon and energy should be focussed on cellular division.

When conditions for growth deteriorate, the cellular level of Spo0A ~ P increases and the *abrB* gene becomes repressed. This frees all the AbrB controlled genes and they can be expressed if their normal regulatory mechanisms are activated. At the same time, the low level of Spo0A ~ P raises the concentration of Spo0A and Spo0F by activating transcription of their genes. This sets the stage for the initiation of sporulation. Higher cellular levels of Spo0A ~ P are necessary to launch transcription of the

sporulation genes spoIIA, *spoIIE,* and *spoIIC* than were required for repression of *abrB* synthesis. Synthesis of the *spoIIA* and *spoIIC* gene products results in the production of the σ^F and σ^E transcription sigma factors, respectively, which allow transcription of those genes specific for cellular compartmentalization and sporulation. This commits the cell to a developmental pathway where temporal gene expression is intimately linked to morphogenetic processes [Driks and Losick, 1991].

Why the cell chose to rely on a single transcription factor, Spo0A, to control this exceedingly complex cellular process remains a mystery. The phosphorelay controlling the synthesis of the active form of this factor must be subject to even more complex controls than we presently imagine.

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