Broadly heterogeneous activation of the master regulator for sporulation in Bacillus subtilis

Arnaud Chastanet1,2, Dennis Vitkupb,2, Guo-Cheng Yuanf, Thomas M. Normanb, Juns Lium, and Richard M. Losicka,2

*Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138; 1Department of Biomedical Informatics, Columbia University, New York, NY 10032; 1Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA 02115; and 2Department of Statistics, Harvard University, Cambridge, MA 02138

Contributed by Richard M. Losick, February 28, 2010 (sent for review February 12, 2010)

A model system for investigating how developmental regulatory networks determine cell fate is spore formation in Bacillus subtilis. The master regulator for sporulation is Spo0A, which is activated by phosphorylation via a phosphorelay that is subject to three positive feedback loops. The ultimate decision to sporulate is, however, stochastic in that only a portion of the population sporulates even under optimal conditions. It was previously assumed that activation of Spo0A and hence entry into sporulation is subject to a bistable switch mediated by one or more feedback loops. Here we reinvestigate the basis for bimodality in sporulation. We show that none of the feedback loops is rate limiting for the synthesis and phosphorylation of Spo0A. Instead, the loops ensure a just-in-time supply of relay components for rising levels of phosphorylated Spo0A, with phosphate flux through the relay being limiting for Spo0A activation and sporulation. In addition, genes under Spo0A control did not exhibit a bimodal pattern of expression as expected for a bistable switch. In contrast, we observed a highly heterogeneous pattern of Spo0A activation that increased in a nonlinear manner with time. We present a computational model for the nonlinear increase and propose that the phosphorelay is a noise generator and that only cells that attain a threshold level of phosphorylated Spo0A sporulate.

At least four feedback loops impinge on the relay. First, 0A→P stimulates the transcription of its own gene (spo0A), acting at a promoter P, that is recognized by RNA polymerase containing σ1. Second, 0A→P stimulates the transcription of the gene (spo0F) for 0F. Third, 0A→P represses the gene (abrB) for AbrB, which in turn represses the transcription of the gene (sigH) for σ1. Finally, 0A→P, via repression of abrB, derepresses the gene (spo0E) for a 0A→P-specific phosphatase Spo0E (0E). The first two pathways (0A→P→spo0A; 0A→P→spo0F) are simple positive feedback loops. The third (0A→P abrB sigH) is also a positive feedback loop in that σ1 directs transcription of kinA, spo0A, and spo0F. Finally, the fourth pathway (0A→P abrB spo0E) is a negative feedback loop in that 0E drains phosphates from the relay (for review see ref. 8).

A striking feature of sporulation is its dichotomy: even under optimal conditions only a portion of the population forms spores (9). The decision of whether to sporulate is dictated by the accumulation of 0A→P. Thus, a tempting hypothesis to explain the bimodality of sporulation was that distribution of 0A→P is itself bimodal because it is subject to a bistable switch (2, 10). The three positive feedback loops governing the synthesis and phosphorylation of 0A were attractive candidates for such a switch.

Here we have revisited the circuitry governing 0A synthesis and phosphorylation. We report that none of the positive feedback loops is rate limiting for the production of 0A. To the contrary, some of the loops seem to be just-in-time mechanisms. Moreover, we show that contrary to earlier thinking, 0A→P activity is not bimodal.

The master regulator for entry into sporulation, Spo0A (0A), accumulates gradually over the first 90 min of sporulation (5) (Fig. S1) and is only active in its phosphorylated form (0A→P) (6). Some genes under its control, such as those involved in biofilm formation and cannibalism, have strong binding sites for 0A→P and are switched ON at low levels of 0A→P. Other genes, such as those for spore formation, have weak binding sites and are only activated when 0A→P accumulates to high levels (5, 7). The accumulation of 0A→P is governed by a regulatory network built around a four-component cascade in which the relay protein Spo0F (0F) is phosphorylated by KinA and other kinases (6, 8). 0F→P, in turn, transfers the phosphate to Spo0B (0B), which transfers the phosphate to 0A (Fig. 1A).
ulatory proteins to the accumulation of OA, starting with AbrB and σH. Transcription of abrB is repressed by OA→P, whereas AbrB, in turn, represses spo0H, the gene for σH (Fig. 1A). Finally, σH directs transcription of spo0A. Indeed, and consistent with earlier reports, the absence of AbrB led to a modest increase in σH levels during exponential phase growth (Fig. 2A). In contrast, the absence of OA caused a marked increase in AbrB levels, resulting in the absence of σH. Thus, OA, AbrB, and σH constitute a positive feedback loop.

To investigate whether this loop contributes to the accumulation of OA at the start of sporulation (hour 0), we carried out the time course experiment shown in Fig. 2A. The results show that the accumulation of σH occurred in parallel with the induction of OA synthesis and, importantly, that the reduction in AbrB levels commenced after OA levels had already started to rise. Thus, the disappearance of AbrB could not be responsible for stimulating OA synthesis. To further investigate the role of AbrB, we took advantage of the fact that spo0A is transcribed both from a vegetative promoter (Pv) responsible for OA synthesis during growth (recognized by σH) and a σH-dependent promoter (Ps) required for sporulation (11). As expected, in a mutant lacking Pv, little or no OA was present during growth, and, as a consequence, AbrB was at higher than normal levels at hour 0 and remained at high levels even at hour 2. Nonetheless, σH levels increased markedly between hours 0 and 2.

Finally, it is known that in addition to repressing abrB, OA→P also turns on the synthesis of an antirepressor AbbA, which prevents AbrB from binding to DNA (12). However, our results show that AbbA is not responsible for derepression of spo0H and hence stimulation of OA synthesis (Fig. S2). We therefore propose the following model (Fig. 2C); during early exponential phase in rich medium, AbrB is at its highest levels (13), thereby repressing its entire regulon. OA→P begins to accumulate by midexponential phase, partially repressing abrB, allowing derepression of certain target genes, such as spo0H, with relatively low affinities for the repressor. Finally, after sporulation commence, OA→P reaches higher levels, inducing AbbA synthesis and further derepression of AbrB targets. As a consequence, targets with high affinities for AbrB are derepressed.

OA→P but Not σH is Rate Limiting for Accumulation of OA. Little induction of the lacZ transcriptional fusion to spo0H (Pspo0H-lacZ) was seen at the same time that σH protein levels were rising (Fig. 2A and Fig. S2), indicating that accumulation of σH at hour 0 is controlled at a posttranscriptional level, as noted previously (14, 15). It therefore remained possible that σH is rate limiting for OA synthesis and that the increase in σH contributes to the timing of induction of OA synthesis. This possibility was ruled out by overproducing σH before the start of sporulation (Fig. S3A); despite the presence of high levels of σH by hour 0, the kinetics of OA→P accumulation were strongly decelerated.

**Fig. 2.** The AbrB/σH pathway contributes negligibly to OA→P accumulation. (A) Kinetics of accumulation of OA, AbrB, and σH during sporulation. Blots were performed using samples of wild-type cells taken at the indicated times before and after suspension in sporulation-inducing synthetic minimal (SM) medium (Right) (25). Samples from cells mutant for spo0A (ΔOA; Abs549) and abrB (ΔabrB; RL3660) were taken immediately before suspension (Left). Equal amounts of protein, as quantified by the Bradford technique, were loaded as verified by the control immunoblot using anti-σH antibodies (Top). (B) σH accumulates to high levels even when AbrB levels remain high. Equivalent amounts of protein from wild-type (wt) or mutant (mut; ΔPspo0A) strains were loaded and analyzed using anti-OA, -σH, -AbrB, and -σH antibodies. (C) Model for the interplay of AbrB, AbbA, OA→P, and σH from growth into sporulation. Arrow indicates transcriptional stimulation. Bars indicate repression or in the case of AbbA inhibition of AbrB protein. Font size conveys relative abundance of proteins. *Unknown posttranscriptional control of σH accumulation.
0A accumulation were unchanged. Next, we hypothesized that the increase in 0A synthesis could result from the feedback loop in which 0A–P stimulates transcription of its own gene. To investigate this, we asked whether the level of 0A–P would increase overall 0A protein levels. First, we observed that the absence of 0E (Fig. S3B), a phosphatase that dephosphorylates 0A–P, sped up the accumulation of 0A, but only modestly. However, using a strain that was both mutant for 0E and that overproduced one of the kinases in the phosphorelay [KinC (16)], we observed a dramatic acceleration of 0A accumulation, reducing the time required to reach maximum accumulation by 50% (Fig. S3B).

We then investigated the effect of increasing 0A–P levels on sporulation by monitoring asymmetric division, the commitment point that requires high levels of 0A–P. Wild-type cells with a polar septum began to appear at approximately hour 1, reaching a peak accumulation at hour 2 (Fig. 3A). The absence of 0E and the overproduction of either KinA or KinC markedly enhanced the rate of appearance of sporangia that had reached the stage of asymmetric division (Fig. 4B). Again, kinase overproduction, especially of KinC, had a more pronounced effect than did elimination of 0E. In addition, the proportion of cells with a polar septum reached almost 100%.

**0A Protein Accumulation Is Not Rate Limiting.** Because 0A–P stimulates the synthesis of 0A, the experiments shown in Fig. 3 did not distinguish between the possibility that 0A protein or phosphate flux through the relay was rate limiting. To discriminate between these alternatives, we took advantage of a 0A mutant (spo0A-up) that is transcribed at higher than normal levels during exponential phase owing to the absence of a repressing element (to be described elsewhere). The immunoblot of Fig. 3B (Inset) shows that cells harboring spo0A-up entered sporulation with more 0A than did wild-type cells. Nonetheless, neither the rate nor the maximum fraction of sporulating cells was measurably higher than for the wild type. Similar results were obtained when 0A was overproduced using the inducible promoter Ppac (Fig. 3C). We conclude that 0A protein is not rate limiting for sporulation.

**Spo0F Is Not Rate Limiting.** One final self-reinforcing cycle that could contribute to the accumulation of 0A–P is stimulation of spo0F transcription by 0A–P. To investigate this, we introduced a P170spo0F fusion into the wild type or a spo0F null mutant and tested the effect of varying isopropyl β-D-thiogalactoside (IPTG) concentrations. Whereas low concentrations of inducer stimulated sporulation in a spo0F mutant, high concentrations inhibited sporulation, possibly owing to the reported substrate inhibition of KinA-mediated phosphorylation at high 0F levels (17). Importantly, none of the tested concentrations supported sporulation at a higher efficiency than observed for the wild type (Fig. 3C). Finally, we considered the possibility that spo0A and spo0F would need to be up-regulated together to increase sporulation efficiency above that observed for the wild type. We therefore created a strain harboring both Pup-0A and P170-spo0F and varied the concentration of IPTG. Again, at no concentration of inducer were we able to observe a higher level of sporulation than for the wild type (Fig. 3C).

Thus, neither 0A nor 0F nor σH are rate limiting for sporulation. Instead, the evidence suggests that the rate-limiting step in the generation of 0A–P is the flux of phosphate through the phosphorelay. We therefore favor the view that the feedback loops governing 0A and 0F synthesis are just-in-time control circuits that are optimally tuned to the requirements of the system for rising levels of phosphorylated Spo0A.

**0A Activity Is Broadly Heterogeneous.** Next, because no feedback loop could account for the postulated bistable switch, we reinvestigated the hypothesis that the distribution of 0A–P is bimodal (10). For this, we carried out fluorescence microscopy using a fusion of the gene for the GFP to the promoter (PspoIII A) for the 0A–P-controlled sporulation gene spoIIA (IIA). The results in Fig. 4A show that fluorescence was broadly heterogeneous, without clear indication of two distinct subpopulations. These results were confirmed by time-lapse flow cytometry.
which revealed a gradual increase in the overall level of 0A–P-directed gene expression but without distinct subpopulations of cells (Figs. 4B and 5A).

That expression of P\textsubscript{H\textsubscript{I}}-\textit{gfp} was heterogeneous was verified by a double-label experiment using cells harboring P\textsubscript{H\textsubscript{I}}-\textit{mCherry} and a fusion of an inducible promoter (P\textsubscript{Hy}) to the gene for mCherry (P\textsubscript{Hy}-\textit{mCherry}). The result in Fig. 4C suggests that cell-to-cell expression levels for P\textsubscript{H\textsubscript{I}}-\textit{gfp} varied much more markedly than for P\textsubscript{Hy}-\textit{mCherry}. Noiseless of \textit{gfp} expression was confirmed by normalizing the data: indeed the coefficient of variation (CV, the ratio of the SD to its mean) of \textit{mCherry} expression was less (0.103) than that of \textit{gfp} (0.353). Furthermore, the low correlation coefficient between the levels of expression of the two fusions (\(R^2 < 0.05\)) indicates that their expression levels varied independently in each cell. If variability in P\textsubscript{H\textsubscript{I}}-\textit{gfp} expression were simply the result of cell-to-cell differences in overall macromolecular synthesis, its level should be highly correlated with P\textsubscript{Hy}-\textit{mCherry}, whose expression is independent of the proteins involved in sporulation. The low correlation coefficient observed between the two reporters, combined with the previously reported low intrinsic noise of this promoter (10), shows instead that the pathway governing the accumulation of \textit{0A–P} is likely responsible for the variability and hence likely to be noisy.

A slightly different result was observed with another \textit{0A–P}-controlled promoter P\textsubscript{IE} (that of \textit{spoIIIE}). Expression of P\textsubscript{IE}-\textit{gfp} commenced later than for P\textsubscript{H\textsubscript{I}}-\textit{gfp} and exhibited a broad distribution, which then coalesced into a high-level expression peak with a tail (120 min) (Fig. 4B). The presence of multiple putative binding sequences for \textit{0A in spoIIIE} promoter raises the possibility...
bility that in this case cooperative interactions among 0A–P bound molecules imparted a heavy-tailed, non-Gaussian distribution of expression levels. Nevertheless, this long-tail distribution does not constitute bona fide bimodality.

**Phosphate Flux Governs the Rate and Timing of 0A–P Activation.**

Next, we took advantage of flow cytometry to analyze the role of phosphate flux in 0A–P activity by monitoring the effect of a spo0E mutation and overexpression of kinA on $P_{IIA}$ gfp expression. Fig. 5A shows the flow cytometry tracings, and Fig. 5B shows corresponding mean fluorescence for each time point so that the changes can be compared over time. We noticed that the increase in 0A–P levels was nonuniform, with a slow stage lasting approximately 1 h, followed by a stage with significantly higher accumulation rate. Additionally, the absence of the 0E phosphatase increased the expression of $P_{IIA}$ gfp, thereby shortening the accumulation stage. Thus, 0E phosphatase in effect creates a delay for the induction of $P_{IIA}$ gfp. Because after the initial slow stage the rate of GFP accumulation (the slope of the curve in Fig. 5B) was largely the same in the 0E mutant as in the wild type, it seems that once the concentration of 0A–P had started to increase rapidly the contribution of the 0E phosphatase became negligible. In contrast, overproduction of KinA significantly accelerated, after a short delay, the rate of GFP accumulation. Finally, when KinA was overproduced in the 0E mutant, the rate of GFP accumulation was accelerated but with less of a delay. We conclude that the 0E negative feedback loop is a timer that delays a stage of fast 0A–P induction, whereas the rate of increase in 0A–P activity is largely determined by phosphate flux.

We also determined the CV for the distribution of 0A–P among cells in the population (Fig. 5C). CV has been used to characterize noise in gene expression (18, 19). We observed that in the wild type the distribution heterogeneity reached a maximum in approximately 45–60 min. Interestingly, both overexpressing kinA and deleting 0E caused heterogeneity to decrease significantly (the CV was 40–60% higher in the wild type than in the overexpression strain and the mutant). From this, we conclude that both 0E and variations in phosphate flux contribute to noise in 0A–P accumulation.

**Computational Model Demands an Increase in the Amount of Relay Proteins During Sporulation.** Finally, we built a computational model for the 0A regulatory network to simulate the kinetics of 0A–P accumulation (assuming that 0A–P was proportional to the mean fluorescence). Modeling was performed using, separately, ordinary differential equations and stochastic equations based on Gillespie’s algorithm (20). To build the model, we used equations describing transcription, translation, protein, and RNA degradation for KinA, 0F, 0B, 0E, and 0A, plus important phosphorylation reactions of the relay (KinA→ 0F, 0F→ 0B, 0B→ 0A), the 0A autoregulatory loop, the dephosphorylation of 0A–P by 0E, and the induction of spo0E by 0A–P. When biochemical parameters were unknown, we relied on previously reported generic parameters (Appendix S1). On the basis of aforementioned results, AbrB and $\sigma^H$ were left out of the model.

In modeling we noticed that to fit the experimental results, we needed to take into account the fact that 0E was overpowered after the initial delay (Fig. 5B). To account for the fast accumulation of 0A–P after 60 min, we had to introduce a significant increase in the amount of KinA. For this, we invoked a linear increase in the rate of kinA transcription, but then other components of the relay became rate limiting, and it proved necessary to increase 0A, 0B, and 0F levels as well. Thus, we introduced similar linear increases in the accumulation of KinA, 0B, 0F, and 0A and optimized the starting point and rates of the increase. The best fit to the experimental data, for both the wild type and the 0E mutant, was achieved when the relay proteins started to increase at approximately 30 min at a rate of approximately 300% per hour (model 1; Fig. 5D). We also attempted to simulate 0A–P accumulation by invoking positive feedback loops in all four proteins instead of a linear increase either on the transcriptional or translational level. To avoid combinatorial fitting of parameters, we assumed the same feedback function for all loops and optimized the feedback strength and sharpness (model 2; Fig. 5E). Both models fit the experimental data reasonably well. It should be noted that although in model 2 the feedback loops are activated only after 0A–P reaches a certain threshold, the network does not reach a new stable steady state after that point. Instead, as in the experimental data, the increasing activity of feedback loops ensures just-in-time increases in the concentrations of the relay proteins for a continuous increase in the 0A–P level.

**Discussion**

Our investigation makes four contributions. First, we provide insight into the function of the feedback loops that govern the synthesis and phosphorylation of 0A. Second, we show that activation of 0A–P occurs in a broadly heterogeneous manner, which, as we propose, arises from noise in flux of phosphate through the relay. Third, we find that 0A activity (0A–P levels) increases in a nonlinear fashion, with an initial slow phase followed by a rapid phase. Fourth, we present time-resolved, computational models predicting an increase in the amount of relay proteins during sporulation that could explain the observed pattern of 0A–P accumulation.

**Function of the Feedback Loops.** Synthesis and phosphorylation of 0A–P is influenced by four feedback loops, which we interpret as follows (Fig. 1B and Fig. S4):

(i) The 0A–P→ AbrB→ $\sigma^H$→ 0A loop is an early switch that is turned on before the period of 0A–P accumulation. Its activation is a prerequisite for 0A–P accumulation, but it does not contribute significantly to the kinetics of 0A–P accumulation.

(ii) The 0A–P→ 0A and 0A–P→ 0F loops are just-in-time circuits that maintain adequate levels of 0A and 0F as 0A–P levels rise, ensuring that neither protein becomes rate limiting. Because 0A accumulates to up to 20,000 molecules per cell (Fig. S1), it would be wasteful to produce a full amount of 0A molecules before they need to be phosphorylated. In the case of 0F, we have shown that it is rate limiting for sporulation at low concentrations and becomes inhibitory at high levels. Therefore, the 0A–P→ 0F loop calibrates the amount of 0F needed as 0A–P levels rise.

(iii) Finally, 0A–P→ 0E→ 0A–P is a negative feedback loop that imposes a delay before 0A–P levels begin to significantly rise. Interestingly, the loop does not influence the rate of 0A–P increase after the delay.

**Broadly Heterogeneous Activation of 0A.** It has been assumed and reported that 0A activation is bimodal, with one or more of the positive feedback loops creating a bistable switch (10, 21). Instead, we find that 0A activity varies greatly from cell to cell and that none of the known loops serve as bistable switches (Fig. S4A). What is then the basis for the heterogeneity? We favor the idea that it is generated by noise in the flux of phosphates through the relay. This noise could arise _stricto sensu_ from the core components of the relay (KinA, 0F, 0B, 0A) or from accessory proteins, such as the phosphatases (Rap and 0E families) known to drain phosphates from the relay (22, 23). Indeed, it is tempting to imagine that the selection for a four-component relay instead of the related, classic two-component system, may...
have been [apart from integrating environmental signals (23)] a way to generate noise.

A Computational Model for 0A Activation. The main conclusions from the FACS data are (i) 0E delays the time at which 0A-P levels start to increase significantly, and (ii) after the delay, the subsequent rate of the 0A-P accumulation is primarily governed by phosphate flux and not by 0E. We attempted to describe this pattern of 0A activation by creating a computational model. A striking conclusion from the modeling simulations was a requirement to significantly increase the amount of relay proteins at some time point after the beginning of sporulation. A slightly better fit for the rise in 0A-P levels was achieved when we assumed that the rates of accumulation of KinA, 0A, 0B, and 0E accelerate owing to positive feedback (model 2). Because no such loops operating at the transcriptional level are known for kinA (recall that σ2, which drives kinA transcription, is not rate limiting) and spotB, thus the model, if true, would imply the existence of an unknown posttranscriptional mechanism to stimulate the accumulation of KinA and 0B.

Biologic Significance. What is the biologic significance that 0A activation is broadly heterogeneous and primarily controlled by the phosphate flux? We suggest that one of the major functions of the phosphorelay, in addition to integrating environmental signals, is to create asynchrony in the time of entry into sporulation. Sporulation requires a high threshold concentration of 0A-P, and only cells attaining this threshold are able to proceed through morphogenesis (Fig. S4). In fact, not all cells in the population reach this threshold and succeed in forming spores. Indeed, and as we have shown, artificially increasing phosphate flux decreases asynchrony and increases the proportion of spore-forming cells. Spore formation and the subsequent process of germination are costly in time and energy. Thus, asynchrony helps to ensure that all cells do not commit to spore formation at once, an advantageous strategy given the vicissitudes of the environment.

In contrast to other systems that generate cell population heterogeneity (such as genetic competence), the rate-limiting factor in the 0A network is not the transcription factor concentration but phosphate flux. This ensures that en route to committing to sporulation B. subtilis constantly monitors environmental conditions through the persistence of this flux. If conditions improve, KinA activity drops and phosphatases, such as 0E, quickly drain phosphate from the network. Thus, 0E plays the role of a persistence indicator, delaying 0A-P accumulation and allowing the majority of the cells to enter sporulation only if adverse environmental conditions persist.

Finally, heterogeneity in 0A-P levels has additional advantages for cells growing to high cell density on surfaces, conditions under which cells exhibit cannibalism—i.e., a delaying tactic for spore formation—and biofilm formation. As noted previously, activation of some target genes, such as those involved in cannibalism and biofilm formation, requires a low threshold concentration of 0A-P. Thus, variation in the rates of accumulation of 0A-P ensures that cells exhibit a variety of behaviors.

Materials and Methods

Media, culture and cloning procedures, and β-galactosidase assay were performed as described previously (24, 25). Immunoblot analyses were performed as described previously (24) using a 10−4 dilution for primary antibody and 2.10−4 dilution of secondary goat anti-rabbit IgG-HRP conjugate (Bio-Rad). Plasmid and strain construction are described in SI Materials and Methods. For assaying sporulation by fluorescence microscopy, membranes were stained using Mitotracker Green FM (Molecular Probes) following a previously described procedure (24), and cell counting was performed using the Metamorph software suite.

For the flow cytometry (FACS) experiment, cell samples were suspended in an equivalent volume of ice-cold PBS buffer and analyzed using a BD LSR II flow cytometer (BD Biosciences) with 30,000 events recorded for each sample. Data were collected with FACS Diva software (BD Biosciences) and analyzed with FlowJo v.8.5.2 and the FCA 2.2 flow cytometry analysis package for Matlab. Stochastic computational simulations were performed using the program DIZZY (26). Kinetics of 0A-P accumulation prediction were obtained by averaging 5,000 individual stochastic simulations. The results of computational modeling were compared with the experimental data by minimizing the rmsd between all simulated and observed time points (from 0 to 120 min) in wild type and in all mutants (see Appendix S7 for details).

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grant GM078990 (to J.S.L. and R.M.L.), National Institute of General Medical Sciences Grant GM079759 (to D.V.), and the National Centers for Biomedical Computing Grant USA4CA121852 (to D.V. and R.M.L.). A.C. was also supported by Agence Nationale de la Recherche Grant 08-JIC-0024-01.

5. Fujita M, Losick R (2005) Evidence that entry into sporulation in Bacillus subtilis is governed by a gradual increase in the level and activity of the master regulator Spo0A. Genes Dev 19:2236-2244.