The Myxococcus xanthus Developmentally Expressed asgB-Dependent Genes Can Be Targets of the A Signal-Generating or A Signal-Responding Pathway

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Functional Myxococcus xanthus A signal-generating and A signal-responding pathways are required for the progression through early multicellular development. To identify genes responsive to these pathways, the expression of eight early developmental genes was analyzed. This examination identified one gene as a target of the A signal-generating pathway and four genes as targets of the A signal-responding pathway.

For a signaling pathway to be fully characterized, all its elements must be identified. The elements will include a signal, a receptor, transducers, and at least a subset of the responsive genes. In certain systems the organism itself generates the signal. An example of this is the Myxococcus xanthus A signaling system required for multicellular fruiting body development. In such cases of autogenous signal generation, two signaling pathways arranged in series must be considered. These are a signal-generating pathway and a signal-responding pathway. Thus, there will be two signals, two receptors, two sets of transducers, and two sets of responsive genes.

The signal-generating and signal-responding pathways are closely connected. The responding pathway is dependent on the presence of the signal. As a result, a defect in the signal-generating pathway will affect the responding pathway. Specifically, the loss of a positive regulator of signal generation will block the expression of gpt (for “generating pathway target”) genes and rpt (for “responding pathway target”) genes. Clearly, responsive genes in both the generating and responding pathways are dependent on the regulators of signal generation.

We have developed a scheme to differentiate gpt and rpt genes in relatively simple systems, such as M. xanthus A signaling, in which signal production is directly proportional to cell density. We hypothesized that gpt gene expression should be directly related to the generation of the signal. In strains defective in signal generation, the gpt genes should not be expressed and addition of the signal should not restore gpt expression. In contrast, rpt gene expression should be directly related to signal activity. In strains defective in signal generation, the rpt genes should not be expressed but addition of the signal should restore rpt gene expression.

We tested this hypothesis on the M. xanthus A signaling system by distinguishing possible gpt and rpt genes from a set of eight genes expressed during early development. The expression of these eight genes is greatly reduced in strains deficient in the production or activity of the Asg regulators of A signal generation (15). These genes are all termed asg dependent. This study classified one gene as a gpt gene and four genes as rpt genes.

Generation of and response to extracellular A signal are required for the progression through early M. xanthus development (11). Extracellular A signal is a specific set of amino acids at an extracellular concentration greater than 10 μM (16). The A signal functions to monitor cell density during starvation, permitting the cells to sense that a minimal cell density (>3 x 10^6 cells per ml) has been reached and development can proceed (17). The A signal is generated, during the first hours of development (15, 16), when proteinases degrade the proteins on the surface of the cell in response to nutrient limitation (21). Each M. xanthus cell generates a fixed amount of A signal, so that the total amount is directly proportional to the number of cells per unit volume (9, 17). This is in contrast to certain other cell density-sensing systems, such as Vibrio fischeri autoinduction, in which the production of the signal is induced by a positive feedback loop (5). Three regulators required for A signal generation are encoded by the unlinked genes (14) asgA (22), asgB (20), and asgC (2). Strains containing mutations in any one of the genes generate A signal at less than 5% of the wild-type level (14) and release extracellular proteins at similarly low levels (21). These mutants are arrested very early in development (8). Certain aspects of development can be restored to asg mutants by the addition of extracellular A signal. This includes the restoration of gene expression and aggregation in all of the asg mutants and partial restoration of sporulation in asgB and asgC mutants (2, 17, 21). These data suggest that A signal is primarily responsible for the asg-dependent developmental arrest.

The asg genes are well characterized. The asgA gene encodes an unusual member of the two-component regulatory systems, in which two domains normally encoded by different genes are fused (22). The asgB gene encodes a putative DNA-binding protein that contains a helix-turn-helix region most similar to the −35 promoter interaction region of major sigma factors (20). The asgC gene encodes the M. xanthus major sigma factor, rpoD (2). Although the regulation may be complex, these genes positively regulate the expression of most developmentally expressed genes (15). The asg gene products are likely to respond directly to starvation by activating the expression of genes that generate the A signal (2, 20, 22). Potential targets include genes involved in protease synthesis and secretion (14). It is expected that a variety of target genes exist and that mutagenesis of most individual genes will not alter signal production significantly.

Previous to this report, the asg-dependent genes were all considered to be in the A signal-responding pathway. This was based on the ability of whole cells to rescue expression of a number of these genes (15). However, the ability of bona fide...
A signal to rescue gene expression was confirmed only for the 4521 gene (16). As more information became available, particularly the observation that the asg genes encoded regulatory proteins (2, 20, 22), it appeared to be possible that some of the asg-dependent genes might be involved in the generation of A signal.

To determine if any of the earliest asg-dependent genes could be classified as gpt genes, the phenotypes of eight strains containing different asg-dependent Tn5 lac fusions were examined. Expression of each fusion begins at a characteristic time between 1 and 8 h after the initiation of development in wild-type cells (Table 1). These fusion-containing strains all form wild-type fruiting bodies (13). It is possible that the Tn5 lac insertions are downstream of the protein-coding regions, that the functions of these genes are redundant, or that the products of these genes are not essential for development. It was expected that gpt genes would be cell density independent, not rescued by extracellular A signal at low cell density, and expressed very early during development. It was expected that rpt genes would be cell density dependent, rescued by extracellular A signal at low cell density, and expressed after 1 h of development.

**Cell density-dependent gene expression.** One of the characteristics that should differentiate the gpt and rpt genes is their cell density dependence. Extracellular A signal is a cell density signal that accumulates in direct proportion to cell density (17). Thus, the gpt genes are expected to be expressed at a constant rate after the initiation of development. This expression should be independent of cell density. In contrast, the expression of rpt genes is expected to increase when extracellular A signal reaches its threshold concentration (10 μM) as the cell density exceeds 3 × 10^6 cells per ml (17). The only well-characterized rpt gene, 4521, exhibits this expression pattern. Its expression is low at densities below 3 × 10^6 cells per ml and then increases dramatically as the density is increased to 1 × 10^7 cells per ml (17) (Fig. 1A).

The cell density-dependent expression of each fusion was tested by the method of Kuspa et al. (17). Briefly, exponentially growing cells, containing one of the eight early asg-dependent Tn5 lac fusions in an asg^+ background, were incubated at different cell densities in starvation buffer. The β-galactosidase activity was measured after 20 h of incubation and normalized to cell number.

The results were striking and separated the genes into two groups (Fig. 1). Six genes, including 4521, showed cell density-dependent increased expression. Expression of five genes (4427, 4442, 4457, 4494, and 4521) was at a relatively low constant level up to cell densities of 5 × 10^6/ml. Expression per cell then increased dramatically as the cell densities were increased to 1 × 10^7/ml. The expression of one gene, 4411, was at an intermediate level up to a density of 5 × 10^6/ml. Expression of this gene also increased as the density was increased to 1 × 10^7/ml. Expression of four genes (4411, 4427, 4457, and 4521) decreased at densities greater than 1 × 10^6 cells per ml (Fig. 1A). Expression of two other genes (4442 and 4494)
Development.
The expression time of each fusion in an 
Tn5 lac fusion in an asg- background was incubated at 2.5 × 10⁸ cells per ml in starvation buffer. The buffer was supplemented with various concentrations of the A-signal amino acid proline or the supernatant from asg+ cells starved at high density. The β-galactosidase activity that had accumulated after 20 h of incubation was compared with the maximum level that fusion reached when the cell density was varied.

The expression of two genes (4273 and 4530) showed a different response to the increase in cell density (Fig. 1C). The expression of these genes decreased as the cell density was increased. Interestingly, the 4273 and 4530 expression patterns are strikingly similar to that of 4521 in a suppressor background, DK6621, in which 4521 expression is A signal independent (10) (Fig. 1C). These data suggest that the expression of 4273 and 4530 is A signal independent.

Phenotypic rescue of gene expression by the addition of A signal. A second characteristic that should differentiate gpt and rpt genes is the phenotypic rescue of gene expression at low cell density by added A signal. At low cell densities, in both asg+ and asg cells, extracellular A signal can not accumulate. Under these conditions, the expression of gpt genes should be relatively unaffected by the addition of A signal, whereas the expression of rpt genes should be restored.

To test the phenotypic rescue of gene expression by the addition of A signal, the method of Kuspa and Kaiser (16) was used. Cultures of cells containing one of the eight early asg-dependent Tn5 lac fusions in an asg- or asg background were incubated at 2.5 × 10⁸ cells per ml in starvation buffer. The buffer was supplemented with various concentrations of the A-signal amino acid proline or the supernatant from asg+ cells starved at high density. The β-galactosidase activity that had accumulated after 20 h of incubation was compared with the maximum level that fusion reached when the cell density was varied.

The addition of A signal phenotypically rescued the expression of a subset of genes (Fig. 2). Four cell density-dependent genes (4442, 4457, 4494, and 4521) were strongly rescued (70 to 300%) by A signal. Surprisingly, two of the cell density-dependent genes (4411 and 4427) were rescued poorly by A signal (a maximum of 30%). The two genes (4273 and 4530) whose expression patterns at various cell densities appeared to be A signal independent were, as expected, poorly rescued by A signal (a maximum of 50%). In most cases, the response to the addition of A signal was similar whether the fusions were in an asg- or asg background. Generally, the phenotypic rescue of gene expression by high levels of proline and that by the wild-type developmental supernatant were similar.

Developmental expression times. A-signal activity increases dramatically at about 1 h after the initiation of development (15, 16). Thus, any gpt genes would be expected to increase expression during the first hours of development. In contrast, rpt genes would be expected to increase expression after 1 h of development. The expression time of each fusion in an asg- background was estimated by measuring at intervals of 15 min, 30 min, or 1 h the β-galactosidase specific activity in cells developing in starvation buffer and on starvation agar plates. The expression of each gene (Table 1) was similar under the two conditions. The expression times of the two genes (4273 and 4530) whose cell density-dependent expression patterns appeared to be A signal independent were strikingly different from each other. The 4530 gene had an expression time of 1 to 1.5 h, and the 4273 gene had an expression time of 4 to 6 h. These results suggest that 4530 may be a gpt gene, but this is unlikely for 4273. Interestingly, 4273 is the only gene of this group of eight in which a Tn5 lac fusion was generated by insertion into a previously identified gene (15). This gene is a spore coat protein gene, tps (4). The regulation of tps expression by the asg genes is likely to be unrelated to A signaling. The expression times of the four rpt genes are between 1 and 4 h. The genes 4411 and 4427, which are cell density dependent and showed poor phenotypic rescue at low cell density by the addition of A signal, have expression times of 2 to 3 h and 6 to 8 h, respectively.

One A signal-generating-pathway target gene and four A signal-responding-pathway target genes are distinguished. These analyses separate the asg-dependent genes into four classes (Fig. 3). One gene, 4530, is a likely target for the A signal-generating pathway. It is surprising that only one putative gpt gene was found. Perhaps the majority of the gpt genes are expressed during growth and development; genes of this
possible that 4411 expression is controlled by a cell-cell contact-mediated signal, whereas 4427 expression may respond to a physiological parameter such as the decrease in oxygen tension that accompanies a higher cell density. Bioassays using wild-type and asg mutant strains containing 4411 or 4427 Tn5 lac fusions as the tester cells should be useful in characterizing these responses.

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