An Early A-Signal-Dependent Gene in *Myxococcus xanthus* Has a σ^{54} -Like Promoter

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Received 10 March 1995/Accepted 5 June 1995

A-signaling plays an essential role in the early stages of *Myxococcus xanthus* fruiting body development. Expression of the 4521 gene, which is regulated at the level of RNA accumulation, depends on starvation and on A-signaling. To identify the *cis*-acting regulatory elements which allow gene 4521 to respond to the nutritional and A-factor signals, the 4521 transcription start site was mapped. The region just upstream of the start site showed sequence similarity to the σ^{54} family of promoters and to the developmentally regulated *mbhA* promoter of *M. xanthus*. A mutational analysis of this region established that the bases which were conserved between the σ^{54} consensus, *mbhA*, and 4521 promoters were also important for 4521 promoter activity. Changes which altered the spacing between two conserved regions centered around positions –14 and –24 abolished promoter activity. In contrast, mutations in a putative –10 region for a σ^{70} -like promoter had little effect on expression of 4521. Despite their similar promoter regions, the regulation of the 4521 and *mbhA* genes was shown to differ with respect to timing of expression and requirement for a solid surface and extracellular signals. This suggests a model in which different activator proteins may be responsible for regulating expression of these two genes.

In response to nutrient limitation in its environment, *Myxococcus xanthus* enters a developmental cycle in which about 100,000 cells aggregate to build a multicellular structure, the fruiting body. Once inside the fruiting body, the individual rod-shaped cells are able to undergo morphological and physiological differentiation to form ovoid, environmentally resistant, and metabolically dormant spores (39). Progress through this developmental program is highly coordinated and involves intercellular communication. Mutational analysis has shown that at least five extracellular signals are required for progress through different stages of the developmental program (6, 9, 26). One of the extracellular signals required early in development is the A-signal, which consists of a mixture of amino acids or small peptides. These signaling molecules have to be present in a narrow concentration range at 1 to 2 h after the onset of starvation and serve as a cell density signal (24). In the absence of extracellular A-signal, as in the *asg* class of mutants, development arrests prior to aggregation of the cells into mounds (22).

To begin to understand how the information conveyed by the A-signal is transduced inside the cell and exerts its normal function in facilitating progress through development, a gene whose expression is dependent on the A-signaling process has been studied. This gene, subsequently called 4521, was identified by a transcriptional fusion of a promoterless *lacZ* gene to its promoter. The fusion was generated by transposition of Tn5*lac* into the transcribed region (20). In an otherwise wild-type cell, the Ω 4521 Tn5*lac* fusion begins to be expressed early in the developmental process, around 1.5 h after the onset of starvation, while in an *asg* mutant, expression fails to increase during development (23). A genetic screen identified two genes, *sasA* and *sasB*, whose products formally act as negative regulators of 4521 expression (16). Mutations in *sasA* and *sasB*

both appear to uncouple 4521 gene expression from the requirement for A-signaling. However, they differ in their effect on the requirement for starvation for 4521 expression. In a *sasA* mutant background, 4521 gene expression is low in vegetative cells, just as in the wild type, and only increases upon starvation. This phenotype is independent of whether any particular *asg* mutation is present; it may be *asgA*, *asgB*, or *asgC* (16). In a *sasB* mutant background, 4521 is already expressed at high levels in vegetative cells, yet expression is increased beyond that level upon starvation. The *sasA* and *sasB* phenotypes are epistatic to the *asg* phenotype, and the *sas* gene products are expected to act downstream of extracellular A-factor in a signal transduction pathway. Because a mutation in the *sasA* gene causes a developmental defect itself which is similar to the defect of an *asgB* mutant, it may define an important component of the A-signal transduction pathway.

The aim of this study was to identify *cis*-acting positive regulatory elements which allow expression of the wild-type 4521 gene only under starvation conditions and in response to A-signaling. The transcription start site of 4521 was mapped and the promoter region was identified through sequence similarity to the promoter of *mbhA*, another developmentally regulated gene of *M. xanthus*. Both promoters were similar to the σ^{54} family of promoters of enteric and other bacteria (25, 42). The functionally important base pairs in the promoter for 4521 were identified by mutation and found to coincide with the bases which were conserved by sequence among σ^{54} promoters.

MATERIALS AND METHODS

Bacterial strains, growth, and development. *Escherichia coli* was grown in LB broth or on LB agar (36) containing ampicillin at 50 μ g/ml, tetracycline at 15 μ g/ml, or kanamycin at 40 μ g/ml when appropriate. *M. xanthus* DK101 and its derivatives were grown at 32°C in CTT broth (12) or on CTT agar (CTT broth plus 1.5% Bacto Agar) supplemented with kanamycin at 40 μ g/ml if appropriate. DK101 (12), which carries *sglAI*, allowing for dispersed growth in liquid cultures, was used as the wild type, and strain DK4322 (23) was used as a control strain which contains the Ω 4521 Tn5*lac* fusion in the DK101 genetic background.

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M. xanthus cells were allowed to develop on a solid surface by growing cells to a density of approximately 5×10^8 /ml, centrifuging and resuspending them in TPM buffer at 1/10 the original volume, and spotting 20- μ l aliquots onto TPM agar plates as described by Kroos et al. (20). To starve cells in suspension, growing cells were concentrated as described above but resuspended in MC7 buffer and shaken vigorously (23).

General molecular biology methods. Molecular cloning and manipulations of plasmid DNA were done by standard methods (36). DNA was sequenced by the dideoxy chain termination method (37) with Sequenase 2.0 as recommended by the manufacturer (United States Biochemical). Single-stranded DNA templates for sequencing and primer extension were prepared as described previously (43). The sequence reported in Fig. 1 was determined on both strands with both dGTP and dTTP in separate sequencing reactions to eliminate compression artifacts. The sequence was assigned GenBank accession number U26450.

RNA preparation and analysis. Total RNA from exponentially growing cells and cells undergoing the early stages of development was prepared by CsCl density centrifugation (40), with modifications as described by Kaplan et al. (16). Slot blot and Northern (RNA) blot hybridizations were carried out by standard methods for the separation of RNA on formaldehyde gels, blotting or transferring RNA directly onto nylon membranes, and probing the blots (16). Primer extension experiments were carried out as described previously (14), with the modifications of Mirel and Chamberlin (31). The primer used was 5' GGCGAGCGGTGCAACAGGCG 3' (named 4521P1) and was synthesized by the PAN Facility (Beckman Center, Stanford University). S1 nuclease analysis was performed by standard methods (36). Single-stranded probes for S1 analysis were prepared either by end labeling restriction fragments (36) or by annealing the end-labeled 4521P1 primer to an appropriate single-stranded template, extending it with Sequenase (United States Biochemical), and cutting the product with an appropriate restriction enzyme to produce a defined 3' end. Both methods of preparing the S1 probes were completed by isolation of the end-labeled antisense DNA strand by denaturing gel electrophoresis (36).

Mutagenesis of the 4521 promoter region. Oligonucleotide-mediated site-directed mutagenesis of the 4521 promoter region utilized the Altered Sites mutagenesis kit (Promega). An approximately 600-bp DNA fragment containing the 4521 transcription start site and upstream regulatory region was cloned as a *Hind*III fragment into the *Hind*III site of pAlter-1 (Promega), generating pIMK116. A 22-bp stretch of DNA in pIMK116 which covered the putative 4521 promoter region was then mutagenized with the spiked oligonucleotide 5' GC CGTGAGCCAAAGCAAGACGCGTCTCGACGCG 3'. The underlined positions correspond to the 22 spiked positions, for which the synthesis reaction contained 4.5% of a mixture of the three incorrect nucleotides. This mixture should, on average, result in the incorporation of 1 mutant base per oligonucleotide molecule (11). The products of the mutagenesis reactions were sequenced with the 4521P1 primer (see "RNA preparation and analysis" above), and all plasmids with a changed sequence were analyzed further.

Introduction of 4521 promoter mutants into *M. xanthus*. The 600-bp DNA fragments containing altered promoter sequences described in the previous paragraph were recloned as *Hind*III-*Bam*HI fragments into the vector pREG1727 (Ron Gill, University of Colorado Health Sciences Center, Denver). Gill's vector contains a multiple cloning site directly upstream of a promoterless *lacZ* gene. In addition, it contains transcriptional terminators upstream of the multiple cloning site to eliminate possible read-through transcription from vector sequences, a fragment with the myxophage Mx8 attachment site *attP* which promotes efficient integration of this plasmid at the *attB* locus in the *M. xanthus* chromosome, and the NPTII gene coding for kanamycin resistance as a selectable marker.

Plasmids carrying the wild-type and mutant 4521 promoters were introduced into *M. xanthus* DK101 by the electroporation method developed by P. Yoderian, University of Idaho (15). Briefly, exponentially growing cells were harvested by centrifugation, washed three times in sterile distilled water at room temperature, and resuspended in 1/100 the original culture volume. Forty microliters of this cell suspension was mixed with 1 to 3 μ l of plasmid DNA and used for electroporation in a 0.1-cm-gap-size cuvette at 0.65 kV, 400 Ω , and 25 μ F, which gave time constants ranging between 7 and 9.5 ms. Following electroporation, the cells were immediately transferred to CTT broth and incubated at 32°C with shaking for 1 to 6 h to allow recovery of the cells and expression of newly introduced genes before plating on selective media.

Developmental β -galactosidase expression in strains containing the wild-type and mutant promoter constructs was assayed as described previously (16).

RESULTS

Mapping the 4521 transcription start site. Expression of the 4521 gene increases in response to starvation and to A-signaling; this increased expression is regulated at the level of RNA accumulation (16). To study the initiation of transcription for 4521 gene expression, the transcription start site was mapped. Because the 4521 gene was first identified by the promoter probe *Tn5lac*, the direction of transcription with respect to the promoterless β -galactosidase gene was known (19, 20). Ap-

FIG. 1. The 4521 promoter region. (A) A partial restriction map of the region upstream of Ω 4521 *Tn5lac* is shown on the top line. Only the relevant *Sac*II and *Bss*HIII sites are indicated. The *Bam*HI site lies within the 5' (left) end of *Tn5lac*, which is represented by the thick line on the upper right. Abbreviations for restriction enzyme sites: S, *Sac*II; E, *Eco*RI; B, *Bss*HIII; A, *Acc*I; Ea, *Eag*I; Ba, *Bam*HI. Bars numbered with Roman numerals represent restriction fragments which were used as probes for low-resolution mapping of the 4521 transcription start site. The hatched box indicates the extent of the sequence reported below. (B) The DNA sequence surrounding the 4521 transcription start site is shown. Broken lines indicate the location of the reported sequence within the region upstream of Ω 4521 *Tn5lac*. The transcription start site is indicated by a bold letter marked +1. The putative promoter elements are boxed. The underlined sequence represents the sequence complementary to the oligonucleotide primer 4521P1, which was used to map the transcription start site. A putative translation start codon and the preceding Shine-Dalgarno sequence (SD) are shown in bold letters. An open reading frame beginning with this start codon continues for at least 106 amino acids (data not shown).

proximately 6 kbp of DNA upstream of the Ω 4521 fusion point had been cloned (16), and a partial restriction map of part of this region is shown on the top line in Fig. 1A. To determine the approximate location of the start site of the 4521 transcript, RNA from wild-type cells was tested for hybridization with restriction fragments derived from DNA upstream of the Ω 4521 fusion point (Fig. 1A). Northern blots were prepared with RNA from vegetatively growing cells and cells that had been allowed to develop on TPM agar for 4 h, and restriction fragments I and V (Fig. 1A) were labeled radioactively to probe the RNA. Probe V hybridized to a developmentally regulated 1.4- to 1.8-kb mRNA species similar to the one identified previously by Kaplan et al. (16). Probe I, derived from DNA upstream of the *Eco*RI site, did not hybridize detectably to any mRNA species present during vegetative growth or early development (data not shown). In an RNA slot blot experiment, restriction fragments II, III, and IV, which derived from DNA downstream of the *Eco*RI site corresponding to restriction fragment V, all hybridized to similar extents to RNA prepared from developing cells but not to RNA prepared from vegetative cells (data not shown). On the basis of these hybridization results, the region extending from the *Eco*RI site to the *Acc*I site corresponding to restriction fragment II of Fig. 1 was expected to contain the 4521 transcription start site.

S1 nuclease protection assays were performed to map more precisely the 4521 transcription start site. A single-stranded DNA probe 5' end-labeled at the *Bss*HIII site and extending

FIG. 2. Mapping of the 5' end of the *4521* transcript by primer extension (A) and S1 nuclease digestion (B). Total RNA of strain DK101 was prepared from growing cells (lanes 1) or cells which were allowed to develop on TPM agar for 4 h (lanes 2). Primer extension and S1 nuclease digestion were performed as described in Materials and Methods. The primer 4521P1 was also used to prime a sequencing reaction on a single-stranded template containing the region of interest. The sequence shown is therefore complementary to the sequence of the mRNA. The transcription start site is indicated by +1.

upstream to the *EcoRI* site (Fig. 1A) was used together with RNA prepared from developing DK101 (wild-type) cells, and a single protected fragment of approximately 280 bp was found (data not shown). On the basis of this result and to maximize the resolving power, an oligonucleotide primer (4521P1) which was complementary to a region estimated from the initial mapping to be no more than 70 bp downstream of the start site of transcription was synthesized. The sequence complementary to 4521P1 is underlined in Fig. 1B. The same oligonucleotide was used both as a primer for extension by avian myeloblastosis virus reverse transcriptase (Fig. 2A) and to prepare a single-stranded end-labeled probe for S1 nuclease mapping (see Materials and Methods) (Fig. 2B). RNA was prepared from wild-type strain DK101 during vegetative growth in CTT broth and after development on TPM agar for 4 h. Primer extension and S1 nuclease digestion identified the same transcription start site when RNA prepared from developing cells was used (Fig. 2, lanes 2). No signal was detected with RNA prepared from growing cells (Fig. 2, lanes 1). Thus, *4521* RNA specifically accumulated early after development is induced by starvation, as had been observed previously (16). In a similar primer extension experiment with RNA prepared from strain DK4322 (23), which carries the $\Omega 4521$ Tn5lac fusion in a DK101 genetic background, the same transcription start site was identified (data not shown). As shown most clearly in Fig. 2A, there is a single transcription start site, presumably indicating a single promoter.

Locating the *4521* promoter. The DNA sequence upstream of the transcription start site of *4521* was scanned for potential promoter sequences. To our knowledge, transcription start sites for eight other genes have been mapped in *M. xanthus* to

FIG. 3. Comparison of the *4521* promoter region with the *mbhA* promoter region and a σ^{54} consensus promoter sequence. The *4521* and *mbhA* promoter regions were aligned by their transcription start site, which is indicated by +1 and is the last base shown in both cases. Sequences identical for the *4521* and *mbhA* promoters are boxed, and sequences which are identical to the σ^{54} consensus promoter (1) are shown in bold type. Abbreviations: Y = C or T; R = A or G; N = A, C, G, or T.

date. These genes consist of the vegetatively expressed genes *vegA* (18), *dsg* (5), and *gufA* (29), the light-inducible *carQRS* operon (29), and the developmentally regulated genes *tps* (13), *ops* (7), *mbhA* (35), and *csgA* (27). All of these genes are regulated differently, and it is probably not useful to look for consensus promoter sequences among them. However, the promoter region of the *mbhA* gene does resemble that of *4521*. *mbhA* encodes myxobacterial hemagglutinin, whose expression is also starvation and A-factor dependent (26, 34). Alignment of the *4521* and *mbhA* genes with respect to the transcription start site showed two conserved sequence elements which are in the same positions upstream of the start site and separated by the same distances (boxed sequences in Fig. 1B and 3). These sequence elements were not centered around canonical -10 and -35 regions as they are found in promoters of the σ^{70} family (10) but were found around positions -14 and -24. This arrangement resembled the σ^{54} family of promoters, with their sequence identities in the regions approximately 12 and 24 bp upstream of the transcription start site (42). Most σ^{54} -like promoters described to date have a GC dinucleotide in their -12 region (30); both the proposed *4521* promoter and the putative *mbhA* promoter have this GC in position -14/-15. Similarly, all σ^{54} -like promoters described to date have a GG dinucleotide in their -24 region. The putative *mbhA* promoter has this GG at -26/-27; the proposed *4521* promoter has AG in these positions (Fig. 3). In addition, the proposed *4521* promoter has the σ^{54} consensus pyrimidine-purine-pyrimidine-purine pattern at -24 (1), a 10-base separation of the -12 and -24 dinucleotides, and consensus TT at -16 (Fig. 3).

Effect of altering bases in the proposed *4521* promoter. To test the relative importance of individual bases in the -7 to -29 region upstream of the *4521* transcription start, bases were randomly mutated in this 22-base interval. This interval covers all of the bases in the proposed σ^{54} promoter, the bases that are identical between the *4521* and *mbhA* upstream regions, and the bases between -7 and -12, which may constitute the -10 region of a σ^{70} -type promoter and whose functional importance might not have been evident through the sequence comparisons. For mutagenesis, a spiked oligonucleotide covering these 22 bases was synthesized. The mutations were introduced into the putative *4521* promoter within an approximately 600-bp fragment of DNA cloned in pIMK116. This fragment includes approximately 200 bp of DNA upstream of the transcription start site, which are sufficient to maintain level and timing of β -galactosidase expression similar to those in strain DK4322, which carries the $\Omega 4521$ Tn5lac fusion (data not shown) (8). Products of the mutagenesis were sequenced, and all mutants were tested for promoter activity by constructing transcriptional fusions to a promoterless *lacZ* gene in plasmid pREG1727 (R. Gill). pREG1727 carries a

FIG. 4. β -Galactosidase expression during development of strains containing *lacZ* under the control of the wild-type and two mutant *4521* promoters. β -Galactosidase specific activity was measured in cells harvested at different times after the initiation of development on TPM agar as described in Materials and Methods. ONP, *o*-nitrophenol.

promoterless *lacZ* gene as well as the bacteriophage Mx8 attachment site, allowing efficient integration of the plasmid at the Mx8 *attB* locus in the *M. xanthus* chromosome. The pREG1727-derived plasmids containing mutated and wild-type control promoter sequences were electroporated individually into *M. xanthus* wild-type strain DK101. Southern hybridizations were used to determine that the plasmids had integrated in single copy at the chromosomal Mx8 *attB* locus. Subsequently, β -galactosidase activity representing the activity of the mutant *4521* promoters was measured at different times of development and compared with the activity of the wild-type promoter fragment at the same locus, whose β -galactosidase activity was measured in the same experiment. Results from a representative experiment are shown in Fig. 4. Activity of the wild-type *4521* promoter, measured as β -galactosidase specific activity, began to increase between 1.5 and 3 h after the initiation of starvation (0 h of development). Among the strains containing mutant promoters which drive β -galactosidase expression, two distinct expression patterns were seen. One is exemplified by a mutation changing G at position -15 to C. In such cases where the mutation abolished promoter activity, β -galactosidase levels never increased beyond levels obtained with the promoterless control construct (see Table 1), which is the background level for these experiments. The second expression pattern is shown by mutations such as the one changing the T residue at position -13 to C. This change resulted in promoter activity which increased β -galactosidase expression with the same timing as that in the wild type but at a lower rate. All other mutant sequences gave time courses of β -galactosidase expression resembling the curves of Fig. 4. On the basis of these similarities of time course, a time point of 5 h after the initiation of starvation was chosen for comparison of mutant and wild-type control activities. At 5 h, β -galactosidase activity produced by the wild-type promoter has typically increased 20- to 30-fold over the background level. The absolute values of β -galactosidase activity and the relative activities of the mutant promoters are shown in Table 1. The relative promoter activities are plotted as a function of base position in Fig. 5 to demonstrate the effect of position.

When bases -19, -20, and -28 of the putative *4521* core

TABLE 1. Activities of mutant *4521* promoters

Promoter assayed	β -Galactosidase sp act at 5 h of development ^a	% Wild-type activity measured in the same expt ^b
Vector only	3.2 $\pm$ 0.2	5 $\pm$ 1
<i>4521</i> Wild type	60.8 $\pm$ 13.9	
<i>4521</i> Mutants ^c		
G -28 A	56.5 $\pm$ 14.8	99 $\pm$ 29
A -27 T	38.1 $\pm$ 3.7	76 $\pm$ 6
G -26 T	3.1 $\pm$ 0.7	4 $\pm$ 1
A -24 T	5.4 $\pm$ 0.7	9 $\pm$ 3
C -23 A	5.1 $\pm$ 1.9	9 $\pm$ 3
G -22 T	7.6 $\pm$ 1.5	13 $\pm$ 2
G -22 Δ	2.3 $\pm$ 0.2	4 $\pm$ 0.2
G -20 T	71.1 $\pm$ 10.1	128 $\pm$ 33
G -20 A	73.1 $\pm$ 5.5	122 $\pm$ 10
T -19 C	54.1 $\pm$ 9.6	76 $\pm$ 20
C -19 Δ	2.8 $\pm$ 0.3	4 $\pm$ 1
G -15 T	1.7 $\pm$ 0.6	2 $\pm$ 1
G -15 C	2.6 $\pm$ 0.5	5 $\pm$ 1
T -13 C	18.7 $\pm$ 6.2	29 $\pm$ 7
T -11 A	84.0 $\pm$ 13.0	147 $\pm$ 24
G -10 T	75.8 $\pm$ 18.4	135 $\pm$ 37
G -9 A	65.2 $\pm$ 21.5	116 $\pm$ 20
G -9 T	53.2 $\pm$ 14.0	102 $\pm$ 46
C -8 T	48.6 $\pm$ 3.7	81 $\pm$ 10
T -7 C	38.7 $\pm$ 7.2	62 $\pm$ 6

^a Expressed in nanomoles of *o*-nitrophenol per minute per milligram of protein (mean $\pm$ standard deviation); each value represents the average of 3 to 10 independent assays in the case of the mutant promoters and 22 independent assays in the case of the *4521* wild-type promoter.

^b The *4521* wild-type promoter construct was included in each separate experiment, and the relative promoter activities were compared with the wild-type activity in that experiment. The average of the percent wild-type activity and the standard deviation were calculated from 3 to 10 independent experiments.

^c For example, mutant G -28 A has a mutation changing position -28 from a G to an A, and mutant G -22 Δ have a mutation deleting G at position -22.

promoter region were mutated, developmental β -galactosidase activity produced by these promoters was nearly indistinguishable from that of the wild type; it ranged from 76 to 128% of the wild-type level. Thus, these bases do not appear to be

FIG. 5. Effect of point mutations on *4521* promoter activity. β -Galactosidase activity of mutant promoter fragments was measured during a time course of development, and the 5-h time point was chosen for comparison of their activities with the wild-type promoter activity levels. The sequence on the x axis at the level of 100% of promoter activity is the wild-type *4521* promoter sequence. Conserved bases between the *4521* and *mbhA* promoters and the *4521* and σ^{54} consensus promoter are indicated as described in the legend to Fig. 3. Relative activity of the promoter mutants is displayed by indicating with the position of the mutant base the level of activity corresponding to the y axis. Levels shown are averages of 3 to 10 independent measurements.

important for promoter activity, and none of them are conserved between the *4521* and *mbhA* promoters or between the *4521* and σ^{54} consensus (Fig. 3). Bases -7 to -11 are in a position corresponding to the -10 region of σ^{70} -type promoters. However, mutations in positions -8 to -11 had little effect on promoter activity; it varied between 81 and 147% of the wild-type activity. A mutation changing the nonconserved T in position -7 to a C had modest effects on promoter activity, reducing it to 62% of that of the wild type. Modest changes of this magnitude may reflect the ability of the promoter DNA to melt and form an open complex with RNA polymerase or the creation of a negative contact between the promoter and the RNA polymerase. Possibilities such as these will require future experiments to resolve. Position -27 differs between the *4521* and the *mbhA* and σ^{54} consensus promoters, but in σ^{54} promoters, it is normally occupied by a highly conserved G. When the A at -27 was changed to a T, activity dropped to only 76% of that of the wild type, which is similar to effects seen with changes in noncritical positions. This result indicates that at least in the *4521* promoter, the identity of the base at -27 is not crucial for promoter activity. In contrast, altering bases within conserved regions produced strong promoter-down phenotypes. Only two of the seven changes in conserved bases resulted in β -galactosidase activities that were significantly above the background level of 5% produced by an *M. xanthus* strain containing only the cloning vector with the promoterless β -galactosidase gene (Table 1). The transition of T to C at position -13 and the transversion of G to T at position -22 resulted in 29 and 13%, respectively, of wild-type β -galactosidase activity. Point mutations at positions -15 , -23 , -24 , and -26 , altering bases conserved between the *4521*, *mbhA*, and σ^{54} consensus promoters, resulted in β -galactosidase activity which was indistinguishable from background levels. The bases at these positions are therefore essential for *4521* promoter activity.

Two single-base deletions at positions -19 and -22 were obtained. These deletions change the spacing between the proposed -12 and -24 regions of the *4521* promoter as well as the angle of base presentation by about 36° , assuming 10 bp per turn of the helix. Both of these deletion mutations abolished *4521* promoter activity, showing that precise spacing and/or angle of presentation between the conserved regions is important.

Despite the similarities in their core promoter regions, *4521* expression and *mbhA* expression are regulated differently. By measuring β -galactosidase expression of a transcriptional fusion of *mbhA* to *lacZ*, Romeo and Zusman established that the *mbhA* gene begins to be expressed after approximately 8 h of development on CF agar and is not expressed in CF liquid (35). In contrast, the $\Omega 4521$ transcriptional fusion to β -galactosidase is expressed by 2 h of development on TPM agar (16, 20). Since CF medium contains 0.015% Casitone, the timing of development and therefore of gene expression may be different from the timing on TPM agar or in MC7 buffer, neither of which contain Casitone or amino acids other than those present in agar. The timing of expression of *4521* and *mbhA* was therefore evaluated under identical conditions. Northern hybridization of RNA prepared from wild-type cells which were allowed to develop on TPM agar showed that while *4521* RNA appeared by 2 h of development (Fig. 6A, lane 2), *mbhA* RNA was only seen to accumulate by 4 h (Fig. 6B, lanes 2 and 4). When RNA prepared from cells developing in MC7 liquid was analyzed, *4521* expression appeared even more strongly by 2 h (Fig. 6A, lanes 3 and 5), while *mbhA* RNA did not accumulate even after 4 h of development in MC7 (Fig. 6B, lanes 3 and 5). Clearly, developmental expression of *4521* and *mbhA*

FIG. 6. Comparison between *4521* and *mbhA* RNA accumulation. RNA was prepared from growing and developing *M. xanthus* DK101 cells, separated on an agarose gel, blotted onto a nylon membrane, and probed with a *4521* probe (A) or an *mbhA* probe (B). The numbers to the left indicate the position of end-labeled simian virus 40 *HinfI* size standards. Lanes: 1, RNA prepared from growing cells; 2 and 4, RNA prepared from cells which were allowed to develop on a TPM agar surface for 2 and 4 h, respectively; 3 and 5, RNA prepared from cells which were allowed to develop in MC7 buffer for 2 and 4 h, respectively.

differs in time and with respect to requirement for a solid surface (agar versus liquid).

DISCUSSION

A-signaling is essential for *M. xanthus* development. In its absence, the *asg* mutants fail to aggregate and sporulate (23). Cells normally respond early to the extracellular A-signal by beginning to aggregate and expressing particular genes, including the *4521* gene (20). To elucidate the pathway by which extracellular A-factor triggers accumulation of *4521* RNA, we have sought to identify *4521* promoter elements. Expression of the *4521* gene is specific for fruiting body development induced by starvation. It is not induced by glycerol, which efficiently induces formation of myxospores without starvation (21). Expression of the *4521* gene is absolutely dependent on A-signaling and is one of the earliest measurable responses of the cells to A-signaling (23). Therefore, the number of steps between extracellular A-factor signaling and expression of the *4521* gene may be small. A functional *4521* gene is not essential for development because a strain carrying the $\Omega 4521$ *Tn5lac* insertion shows no obvious developmental defects (20).

The same unique transcription start site of *4521* was identified by primer extension and by S1 nuclease mapping experiments. An upstream region from -12 to -26 which showed similarity to the σ^{54} consensus promoter as well as to the putative promoter of *mbhA* was found. Mutagenesis of this region established its importance for developmentally regulated expression of a transcriptional fusion of the *4521* promoter to *lacZ*. To our knowledge, this study reports the first *in vivo* functional characterization of a promoter in *M. xanthus*.

Several lines of evidence support the hypothesis that the *4521* gene is transcribed by a σ^{54} -containing RNA polymerase holoenzyme. First, mutagenesis of the suggested *4521* promoter strongly supports a requirement for the σ^{54} consensus base pairs. Site-directed mutagenesis of several σ^{54} -transcribed promoters has been reported. When the base equivalent to position -28 in the *4521* promoter is mutagenized in either the *Klebsiella pneumoniae nifH* (2) or the *Caulobacter crescentus*

flbG (32) promoters, the resulting promoter activity is essentially that of the wild type or only slightly decreased, just as in the case of the 4521 promoter. Mutations in the position -27 equivalent, which is G in the σ^{54} consensus but is replaced by an A in the 4521 wild-type promoter, have no activity in the case of the *K. pneumoniae nifL* promoter (17) but result in approximately 40% of wild-type activity in the *Azorhizobium caulinodans nifA* promoter (41). In addition, changing the bases in the equivalent -28/-27 positions in the *Bradyrhizobium japonicum fixR nifA* promoter from TG to CT does not appear to affect promoter activity, indicating that position -27 may not be important for activity of a subset of σ^{54} -type promoters. When the consensus G in position -26 of 4521 was changed to a T, β -galactosidase activity fell to background levels, and an equivalent mutation in the *K. pneumoniae nifL* promoter has the same effect (17). Similar to the case of the *nifH* and *nifL* promoters of *K. pneumoniae* (2, 3, 17), when bases in the nonconserved spacer region between the -12 and -24 regions were mutated, promoter activity was not decreased. Mutation of the highly conserved G residue in position -15 resulted in a very strong promoter-down phenotype in the 4521 promoter. When corresponding base pairs are mutated in the *K. pneumoniae nifH* or the *A. caulinodans nifA* promoters, a similarly strong promoter-down effect is seen. In contrast, mutating bases between -7 and -11 upstream of the 4521 start, which would be expected to constitute the -10 region of a promoter of the σ^{70} family, had little or no effect on promoter activity. The activity of σ^{54} -type promoters is unusually sensitive to changes in the spacing between the -12 and -24 regions. Where it has been tested, a spacing of precisely 10 bp between GG at -12 and GC at -24 has been shown to be essential (2, 32), while the spacing between the -35 and -10 regions of the σ^{70} family of promoters appears to be less critical since it ranges from 16 to 18 bp (10). The two mutations which delete a base pair between the -12 and -24 elements of the 4521 promoter abolished promoter activity.

Another indication that the 4521 promoter may be transcribed by a σ^{54} -containing RNA polymerase holoenzyme is that fully regulated 4521 gene expression requires 146 bp of DNA upstream of the transcription start site (8). This suggests that binding of other factors in addition to the RNA polymerase holoenzyme may be required in this region. Gulati et al. have found that a protein present in crude cell extracts of *M. xanthus* is able to bind to a fragment of DNA extending from 146 bp upstream to 24 bp downstream of the 4521 transcription start site. This binding activity is dependent on the presence of DNA between -146 and -90 upstream of the transcription start site (8). In all systems investigated, a σ^{54} promoter requires the binding of an activator protein to sequences upstream of the immediate promoter region for activity (25, 30). The σ^{54} -containing RNA polymerase holoenzyme by itself is able to bind to the promoter region and to form a closed complex. However, it is unable to proceed to the open complex form and to initiate transcription in the absence of an activator protein (33, 38).

Taken together, the data argue that the promoter for 4521 belongs to the σ^{54} family and not to the extended σ^{70} family. Since 4521 gene expression is regulated in response to two environmental signals, starvation and A-factor, one or both of these regulatory inputs may act through the production or activation of an activator protein. A gene coding for a σ^{54} homolog in *M. xanthus* has been identified (16a).

The significant similarity between the core promoter regions of the *mbhA* and 4521 genes suggests that the same RNA polymerase holoenzyme may be used for transcription of both genes. However, regulation of these genes differs in several

aspects. Expression of the hemagglutination activity of the *mbhA* gene product during development is both A-signaling and D-signaling dependent (26), while expression of 4521, although dependent on A-signaling (23), is not dependent on D-signaling (4). As shown here, *mbhA* RNA begins to accumulate approximately 4 h after cells are placed under starvation conditions, at least 2 h after 4521 RNA begins to accumulate (16). This is approximately 4 h before β -galactosidase from the *mbhA-lacZ* transcriptional fusion begins to be expressed on CF agar (35). In addition, while 4521 RNA accumulated even when the cells were subjected to starvation conditions by shaking in MC7 buffer in the absence of a solid surface, accumulation of *mbhA* RNA required the presence of a solid surface (Fig. 6), confirming the results of Romeo and Zusman (35).

How can these differences between 4521 and *mbhA* in timing and conditions for gene expression be reconciled with the strong similarities in their putative promoter sequences? Like 4521 (8), the *mbhA* gene requires DNA upstream of the core promoter region for developmentally regulated expression (35). If both promoters were recognized by a σ^{54} -containing RNA polymerase holoenzyme, the differential regulation of the 4521 and *mbhA* genes could be due to binding of different activator proteins to the region upstream of the core promoters to activate gene expression. These hypothetical activator proteins may themselves be expressed or activated in response to different signals from the cell's external or internal environment, such as the A- and D-signals. In *Bacillus subtilis*, endospore formation depends on a cascade of sigma factors which coordinate and differentiate the spatial and temporal expression of genes in the mother cell and forespore compartments during development (28). In *M. xanthus* fruiting body development, a series of different activator proteins for the same σ^{54} RNA polymerase holoenzyme could be responsible for differential activation of gene expression.

ACKNOWLEDGMENTS

We thank Ron Gill for providing pREG1727, Heidi Kaplan for communicating results prior to publication, Craig Stephens for advice, and Mitch Singer and Lotte Sogaard-Andersen for discussions, comments, and suggestions on the manuscript.

This work was supported by Public Health Service grant GM23441 from the National Institutes of Health to D.K.

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