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Transcription of the Myxobacterial Hemagglutinin Gene Is Mediated by a σ^{54}-Like Promoter and a *cis*-Acting Upstream Regulatory Region of DNA

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Myxobacterial hemagglutinin (MBHA) is a major developmentally induced protein that accumulates during the period of cellular aggregation of the fruiting bacterium *Myxococcus xanthus*. In this study, DNA sequences mediating the transcriptional regulation of *mbhA* have been identified. Examination of nucleotide sequences upstream of the start site for *mbhA* transcription has indicated a region of DNA that bears strong homology to the consensus sequence for promoters recognized by the σ^{54} holoenzyme form of RNA polymerase of *Escherichia coli* and other eubacteria. Deletion of this sequence completely abolished *mbhA* transcription. Additionally, a *cis*-acting DNA element, affecting the efficiency of *mbhA* transcription, has been mapped within a region of DNA 89 to 276 nucleotides upstream of the σ^{54}-like sequence. Transposon insertions, mapping within the *cis* element, drastically reduced *mbhA* transcriptional activity. These observations suggest that transcription of *mbhA* requires a productive interaction between a form of RNA polymerase that recognizes a σ^{54}-like sequence and a transcriptional activator that binds to DNA sequences upstream of the *mbhA* promoter.

The gram-negative bacterium *Myxococcus xanthus* is unusual among procaryotes in the degree to which it exhibits multicellular phenomena throughout its life cycle (19, 45, 49). During vegetative growth, cells move by gliding motility in groups and are capable of cooperatively digesting other microorganisms as well as a variety of types of particulate organic material. When levels of available nutrients fall below what is necessary for sustaining vegetative growth, a program of primitive development is initiated: individual cells move into aggregation centers and subsequently undergo a process of morphogenesis culminating in the differentiation of the rod-shaped vegetative cells into spherical, environmentally resistant resting cells called myxospores. Raised aggregates containing mature myxospores are referred to as fruiting bodies.

Much of the interest in the myxobacteria has been directed toward the elucidation of the environmental cues that initiate development and the search for the genes and gene products that mediate it. One of the first development-associated gene products to be studied was the myxobacterial hemagglutinin (MBHA; 50). This lectinlike protein is synthesized at barely detectable levels during vegetative growth but is abundantly synthesized during development (8, 9). The sequence of the *mbhA* gene indicates that the MBHA polypeptide has a tetrameric structure composed of four homologous domains (40). Following its synthesis in the cytoplasm, MBHA is targeted to the periplasm and is apparently also associated with the cell surface, where it has been detected at the cell poles by immunofluorescence microscopy (33).

The phenotypic analysis of *mbhA* mutants has suggested that in certain environments, such as media low in magnesium, MBHA may play an important role in mediating the process of cellular aggregation. However, under the normal conditions of laboratory culture, its role in this process appears to be subtle; mutants unable to synthesize MBHA appear to be delayed but not blocked in fruiting body formation (41).

The ease of the hemagglutination assay has made MBHA a convenient marker for development. Studies involving mutants blocked at different stages of development suggest that the expression of MBHA is dependent on events which occur earlier in development; strains carrying mutations in *asg* (22) or *dsg* (7) are blocked in a very early stage of development and do not synthesize MBHA (25). However, there is very little known about the precise molecular events that mediate the expression of MBHA. In this study we focus our attention on the transcriptional signals of *mbhA*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Relevant bacterial strains are listed in Table 1, and plasmids are listed in Table 2.

Plasmid pJR114x1 was constructed by oligonucleotide-directed mutagenesis of pJR114, using a commercial kit (Muta-Gene; Bio-Rad, Richmond, Calif.). A 46-mer deoxyoligonucleotide primer was synthesized with the sequence 5′-CGGCTCCGCGCAGCCGCGAACTCGAGTCGTCGT GAGAGGGACTTCC-3′. The 5′ and the 3′ regions of this primer each contain 20 nucleotides of perfect homology with the *mbhA* sequences flanking the σ^{54} consensus sequence; the central six nucleotides encode an *Xho*I site and are mismatched with the σ^{54}-like sequence. Mutagenesis with this primer effected the replacement of the σ^{54}-like sequence (nucleotides 334 to 349 of Fig. 4A) with the *Xho*I hexamer.

Plasmid pJR110D was constructed by digestion of pJR110 with *Sst*I and *Pst*I, removal of the 3′ protruding termini with the Klenow fragment of DNA polymerase I, and recircularization of the plasmid by treatment with T4 DNA ligase. Other plasmids are described in Table 2 and were constructed by standard techniques (4, 26).

Bacterial culture conditions. *M. xanthus* was grown in Casitone yeast extract (CYE; 6) medium either in liquid broth with shaking at 30°C or on CYE plates containing 1.5% agar (Difco Laboratories, Detroit, Mich.). For induction of

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TABLE 1. *M. xanthus* strains

Strain	Relevant phenotype	Comment	Source or reference
DZF1		Wild-type strain FB	31
DZF3436	Kan^r	Tn*5*Ω103 in *mbhA*	41
DZF3598	Tet^r Kan^s	Tn*5*-132Ω103 in *mbhA*	P1::Tn*5*-132 transduction into DZF3436
DZF3561	Kan^r	Tn*5*-*lac*Ω156 in *mbhA*	21, 41
DZF3600	Kan^r	Tn*5*Ω20 in the region upstream of *mbhA*	P1::Tn*5* transduction into DZF1
DZF3601	Kan^r	Tn*5*-*lac*ΩRS2 in the region upstream of *mbhA*	P1::Tn*5*-*lac* transduction into DZF1
DZF3602	Kan^r	Tn*5*Ω41 in the region upstream of *mbhA*	P1::Tn*5* transduction into DZF1
DZF3603	Kan^r	Tn*5*Ω101 in the region upstream of *mbhA*	P1::Tn*5* transduction into DZF1
DZF3604	Kan^r	Tn*5*Ω238 in the region upstream of *mbhA*	P1::Tn*5* transduction into DZF1
DZF3566	Kan^r Tet^s	Δ5-2 deletion in *mbhA*; gene replacement	41
DZF3606	Kan^r Tet^r	*mbhA* merodiploid	P1-mediated transduction of pLJS49-112 into DZF3598
DZF3607	Kan^r Tet^r	*mbhA* merodiploid	P1-mediated transduction of pLJS49-114 into DZF3598
DZF3608	Kan^r Tet^r	*mbhA* merodiploid	P1-mediated transduction of pLJS49-114X1 into DZF3598

development, vegetative cultures were grown to a cell density of 2×10^8 to 4×10^8 cells per ml, harvested, and resuspended in TM buffer (0.01 M Tris hydrochloride [pH 7.5], 0.008 M $MgSO_4$) to a cell density of 4×10^9 cells per ml. This concentrated cell suspension was spread on 100-mm-diameter CF agar plates (16) at a concentration of 2.5×10^9 cells per plate, and the plates were incubated at 34°C.

P1 transduction was performed essentially as described by O'Connor and Zusman (37); Tn*5* mutagenesis was done as described by Downard et al. (11). All transduced strains used in this study were verified by Southern blot hybridization (data not shown).

β-Galactosidase assays. Cells were harvested from CF agar plates with a razor blade, resuspended in TM buffer, and disrupted by sonication. β-Galactosidase was assayed as described by Miller (28). Specific activity was expressed as nanomoles of product formed per minute per milligram of protein.

RNA isolation. Vegetatively growing cells were harvested by centrifugation; developmental cells were harvested by scraping CF agar plates with a razor blade. The cell paste was solubilized with UNSET buffer (8 M urea, 0.15 M NaCl, 0.1 M Tris hydrochloride [pH 7.5], 0.001 M EDTA, 2% sodium dodecyl sulfate), and RNA was purified as described previously (13, 14). The concentration of the purified RNA was determined spectrophotometrically.

Northern (RNA) blot analysis. Purified RNA was treated with glyoxal and fractionated on a 1.4% agarose gel as described previously (26) and then transferred to a modified nylon membrane (Nytran; Schleicher & Schuell, Keene, N.H.), using protocols provided by the manufacturer. The membrane was probed with ^{32}P-labeled pJR100, and hybridization was visualized by autoradiography.

Primer extension analysis. A synthetic 36-mer deoxyoligonucleotide was synthesized with the 5′-TCCTTGAGTCG AGGTTGGACTGACGCAACTGCGGGC-3′. The 36-mer was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. Five picomoles of labeled oligonucleotide was hybridized with 50 μg of RNA in a buffer containing 80% formamide, 0.4 M NaCl, 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), and 0.001 M EDTA for 16 h at 30°C. The hybridized nucleic acids were precipitated with ethanol and treated with reverse transcriptase essentially as described previously (4).

Western immunoblot analysis. Cells were scraped from CF agar plates after 20 h of development, resuspended in TM buffer, and disrupted by sonication. Proteins (10 μg) were fractionated by electrophoresis through 12.5% polyacrylamide gels (24), transferred to nitrocellulose membranes, and incubated with anti-MBHA antibody and ^{125}I-labeled protein A (4).

DNA sequencing. Nucleotide sequences were determined

TABLE 2. Plasmids

Plasmid	Vector	Drug resistance marker(s)	Comments
pJR99	pUC9	Amp^r	869-bp *Hin*cII fragment from pJR100 (41) cloned into the *Hin*cII site of pUC9 (36)
pJR100	pUC9	Amp^r	3.2-kbp *Sal*I fragment of *M. xanthus* DNA cloned into the *Sal*I site of pUC9 (41)
pJR110	pUC9	Amp^r	860-bp *Sal*I fragment containing the 3′ portion of *mbhA* cloned into the *Sal*I site of pUC9
pJR110Δ	pUC9	Amp^r	Deletion of a 450-bp *Sst*I-*Pst*I fragment from pJR110 effecting the deletion of DNA sequences downstream of *mbhA* region
pJR114	pTZ18 (27)	Amp^r	*Eco*RI-*Sal*I fragment from pJR99 ligated with *Eco*RI-*Sal*I-cleaved pJR110Δ to generate a complete *mbhA* region
pJR114X1	pTZ18	Amp^r	Deletion of *mbhA* promoter by oligonucleotide-directed mutagenesis
pLJS49-112	pLJS49	Amp^r Kan^r	*Eco*RI-*Hin*dIII *mbhA* fragment from pJR112 (41) ligated with *Eco*RI-*Hin*dIII-cleaved pLJS49 (46)
pLJS49-114	pLJS49	Amp^r Kan^r	*Eco*RI-*Hin*dIII *mbhA* fragment from pJR114 ligated with *Eco*RI-*Hin*dIII-cleaved pLJS49
pLJS49-114X1	pLJS49	Amp^r Kan^r	*Eco*RI-*Hin*dIII *mbhA* fragment from pJR114X1 ligated with *Eco*RI-*Hin*dIII-cleaved pLJS49

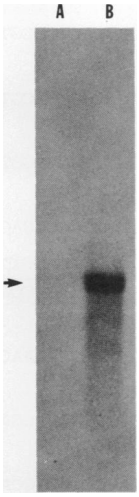

FIG. 1. Northern blot analysis of *mbhA* mRNA. RNA was purified from vegetative (A) cells or developmental (B) cells of *M. xanthus* DZF1. Developmental cells were harvested 18 h after induction of development of CF agar. RNA (10 μg) was treated with glyoxal and fractionated on a 1.4% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled pJR100 as described in Materials and Methods.

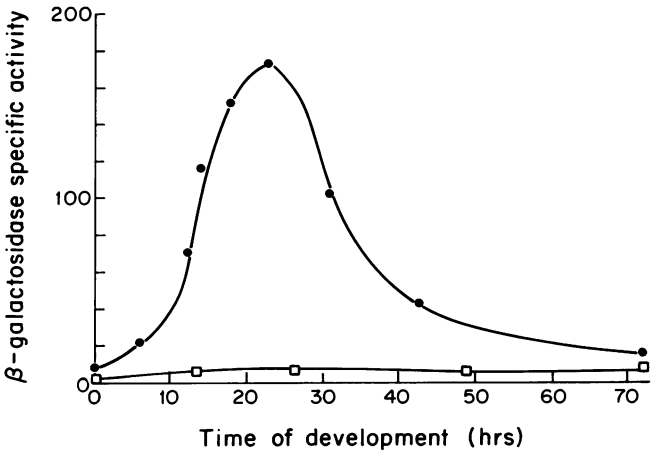

FIG. 2. Developmental expression of an *mbhA-lacZ* transcriptional fusion. *M. xanthus* DZF3561 was grown vegetatively, harvested, and either plated for development on CF agar (●) or shaken in CF broth (□) at 34°C; cells were harvested at the indicated times for determination of β-galactosidase activity.

by the dideoxy-chain termination method of Sanger et al. (42). Sequencing reactions were done with Sequenase T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, Ohio), using protocols provided by the manufacturer.

Nucleotide sequence accession number. The sequence shown in Fig. 4A has been assigned GenBank accession number M60079.

RESULTS

Transcription of *mbhA* is regulated during development. Two lines of evidence indicate that the synthesis of MBHA is regulated at the level of transcription. (i) Figure 1 shows the results of a Northern blot analysis performed on RNA extracted from vegetative cells of *M. xanthus* and from cells which were developing on CF agar. The results clearly demonstrate the absence of a detectable mRNA transcript for *mbhA* during vegetative growth and the presence of an abundant *mbhA* transcript during development. (ii) Figure 2 shows the use of an *mbhA-lacZ* transcriptional fusion (21), which was monitored throughout development as an independent method of estimating the transcriptional activity of *mbhA* during fruiting body formation. There was good agreement between the levels of β-galactosidase activity seen in the strain containing the transcriptional fusion (DZF3561) and the levels of hemagglutinating activity observed in the wild type, DZF1 (8). We will show elsewhere that the *mbhA* mRNA is extremely stable during development, suggesting that modulation of mRNA half-life may be a factor contributing to the increased levels of *mbhA* mRNA observed during development (41a). However, these same studies have indicated that the *mbhA-lacZ* mRNA is not stabilized by the fusion and is extremely unstable during development. Furthermore, it has been shown that the stability of β-galactosidase activity is slightly decreased during development (48). These considerations strongly suggest that the increased β-galactosidase activity displayed by the *mbhA-lacZ* fusion strain reflects the transcriptional activation of *mbhA* and that the synthesis of MBHA during development is determined at least in part by the rate of *mbhA* transcription.

It should also be noted that fruiting body formation was slightly delayed in DZF3561 as a result of the Tn*5-lac* insertion in *mbhA* (41). However, the peak of β-galactosidase activity still correlated with the same aggregation stage of development normally associated with MBHA production in the wild-type strain, DZF1 (41).

Figure 2 also shows that when cells were transferred from rich medium to starvation conditions in liquid medium, only very small levels of induction of *mbhA* transcription were observed. Thus, *mbhA* transcription was efficiently induced only when cells were starved on a solid surface; this result paralleled the previously described regulation of MBHA polypeptide (8).

Localization of the 5′ end of the *mbhA* mRNA. Low-resolution mapping of the 5′ end of the *mbhA* mRNA was accomplished by an RNase protection mapping protocol (data not shown). The results of these experiments suggested a single region of transcriptional initiation and allowed the design of a chemically synthesized DNA oligonucleotide which was used for high-resolution mapping by primer extension analysis. Developmental RNA from DZF1 gave a clear signal in this assay (Fig. 3, lane 2). The extended product was specific for *mbhA* mRNA, since it was absent in a strain carrying an *mbhA* deletion (Fig. 3A, lane 1).

Since we have found that the *mbhA* mRNA has an unusually long half-life during development ($t_{1/2}$ = 2.5 h; 41a), we considered the possibility that some form of processing of the 5′ end of the mRNA occurs to mediate this unusual stability. We therefore also mapped the 5′ end of the mRNA from strain DZF3436, in which transposition of a Tn*5* element into *mbhA* results in the production of two truncated derivatives of the *mbhA* mRNA which have a much shorter half-life ($t_{1/2}$ = 6 min; 41a). The position of the 5′ end of the mRNA in this strain was found to be identical to that of the wild-type strain (Fig. 3 lane 3).

Figure 4A shows the nucleotide sequences of the DNA upstream of the 5′ end of the *mbhA* mRNA; analysis of the sequence indicated a region which bears strong homology to the promoters recognized by the σ^{54} holoenzyme form of

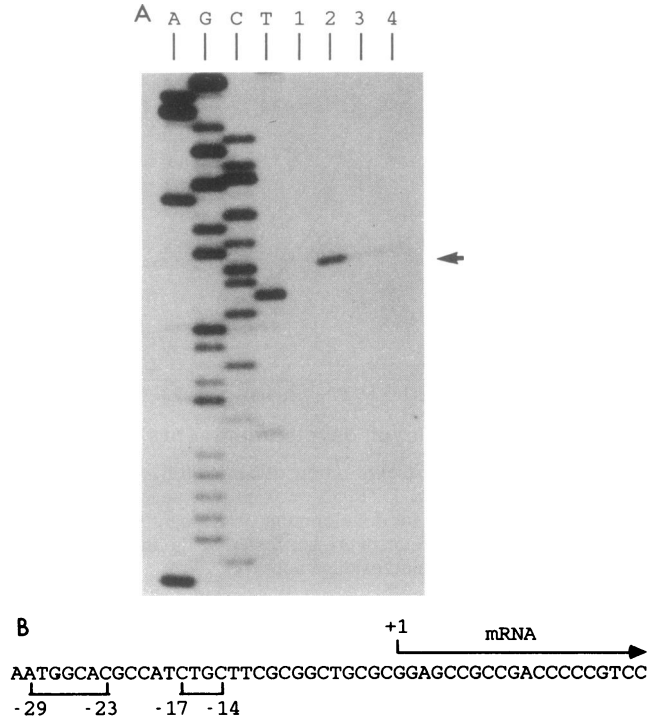

FIG. 3. (A) Primer extension analysis. RNA was purified after 18 h of development on CF agar from DZF3566 (*mbhA* deletion) (lane 1), DZF1 (wild type) (lane 2), DZF3436 (*mbhA*::Tn*5*) (lane 3), and DZF3601 Tn*5-lac* upstream of *mbhA*) (lane 4) and used to prime reverse transcription as described in Materials and Methods. The ^{32}P-labeled primer was also used to prime dideoxy sequencing reactions for size comparison, using the dideoxy nucleotides indicated (lanes A, G, C, and T). The Arrow indicates the position of the *mbhA* mRNA 5′ end. (B) Position of the mRNA start site in relation to the sequence. The extended product was 64 nucleotides in length; the reverse transcription primer was complementary to nucleotides 391 to 426 (see Fig. 4A).

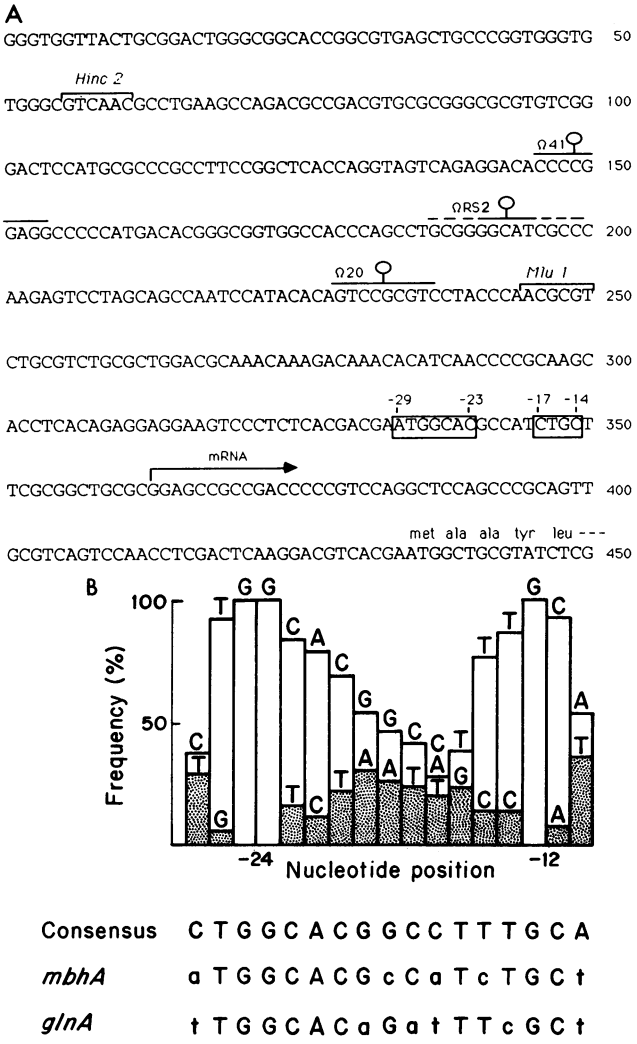

FIG. 4. (A) Nucleotide sequence of the *mbhA* upstream region. The locations of the Tn*5* insertions were determined by DNA sequencing of the transposon-*mbhA* junctions for each of the Tn*5* insertions used in this study. The 9-bp duplication caused by the transposition of Tn*5* is indicated above the sequence for each of the insertions. The position of the Tn*5-lac* insertion (ΩRS2) was estimated from the mobility of restriction endonuclease-generated fragments from this construct. The nucleotide sequence from positions 248 to 450 has previously been published (40). The σ^{54}-like sequences are boxed, and the mRNA start site is indicated with an arrow. (B) Comparison of the *mbhA* σ^{54}-homologous sequence with similar consensus sequences of 64 promoters from 22 species as analyzed by Morett and Martin (30). The *glnA* promoter from *E. coli* (15) is also shown for reference.

RNA polymerase in *Escherichia coli* and other eubacteria (Fig. 4B). The spacing between the σ^{54}-like homology and the initiating nucleotide of *mbhA* mRNA was 13 nucleotides, corresponding to an appropriate distance between these elements (15, 23, 30).

Tn*5* mutagenesis of the *mbhA* promoter region. *M. xanthus* strains containing single-copy insertions of the transposon Tn*5* in the putative *mbhA* promoter region were monitored for levels of MBHA polypeptide synthesis during development by Western blot analysis (Fig. 5). It was found that Tn*5* insertions mapping between 105 and 185 nucleotides upstream of the σ^{54} consensus sequence reduced but did not abolish *mbhA* gene expression (lanes 3 to 5), while insertions that mapped approximately 2 or 1 kbp upstream had no significant effect on MBHA levels (lanes 1 and 2). The *mbhA* mRNAs synthesized in strains DZF3600 to DZF3602 were also analyzed by Northern blot and yielded similar results (shown only for DZF3601 in Fig. 6D). In these strains, a reduced amount of an mRNA the same size as the wild type was produced, and development on CF agar was wild type in appearance and followed normal kinetics. The 5′ end of the *mbhA* transcript from one of these strains, DZF3601, was also mapped and was found to be identical to that of the wild type by primer extension analysis (Fig. 3A, lane 4), demonstrating that the residual gene expression detected in this strain was the result of transcription from the *mbhA* promoter rather than from spurious transcription induced by the Tn*5* insertion. Thus, sequences upstream of the σ^{54} consensus sequence are required for the efficient utilization of the *mbhA* promoter. The requirement for such sequences is a general feature of σ^{54} promoters (23).

Merodiploid analysis of *mbhA* transcription. To further define the *mbhA* promoter region, we analyzed three merodiploid strains of *M. xanthus*. Each of the strains contained a Tn*5*-132 element (5) in the normal chromosomal copy of *mbhA* to eliminate the full-length *mbhA* transcript generated

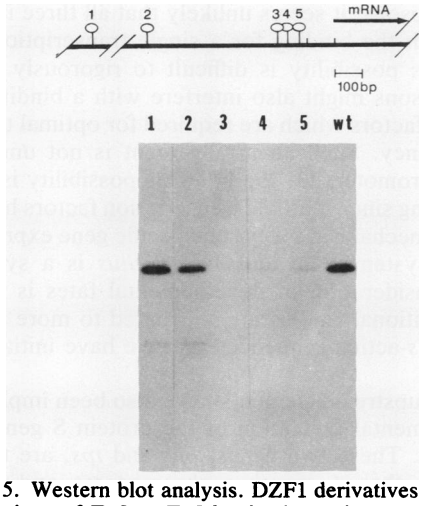

FIG. 5. Western blot analysis. DZF1 derivatives containing single insertions of Tn*5* or Tn*5-lac* in the region upstream of *mbhA* were monitored for MBHA synthesis during development. The individual transposon insertions are indicated in the diagram and the autoradiogram as follows: 1, DZF 3604; 2, DZF3603; 3, DZF3602; 4, DZF3601; and 5, DZF3600.

from this locus. Transcription of the Tn*5*-disrupted *mbhA* generated two truncated transcripts, 450 and 600 nucleotides in length, which could be detected on Northern blots and served as internal standards (Fig. 6A to C). Three different *mbhA* constructs, containing different upstream regions, were then transduced into the *M. xanthus* chromosome with the vector pLJS49 (46). This vector is useful for targeting

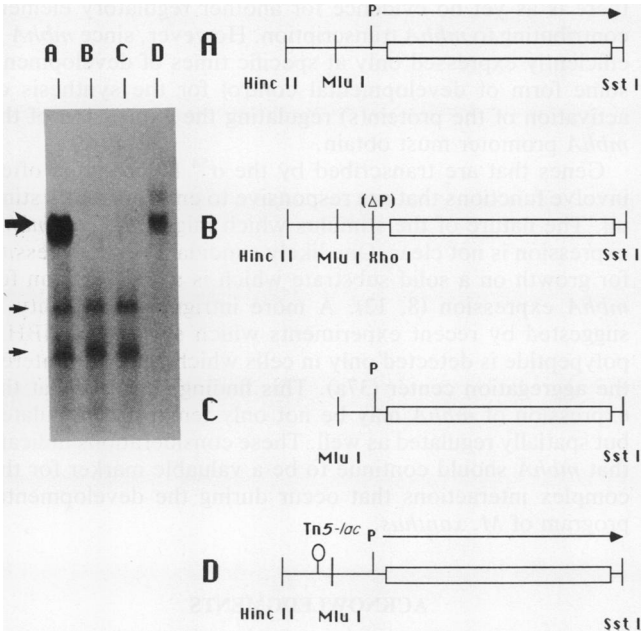

FIG. 6. Effect of alterations in the upstream region of *mbhA* on levels of transcription. RNA was purified from DZF3607 (A), DZF3608 (B), DZF3606 (C), and DZF3601 (D) after 24 h of development on CF agar at 34°C, and 20 μg was analyzed by Northern blot hybridization. The large arrow indicates the position of the wild-type *mbhA* transcript; small arrows indicate the positions of the truncated *mbhA*::Tn*5* transcripts.

cloned DNA to the bacterial attachment site for myxophage Mx8 by site-specific recombination. Following transduction, *mbhA* transcription from each strain was assayed by Northern blot analysis (Fig. 6). Plasmid pLJS49-114, which contains the structural gene for MBHA and 307 bp upstream from the transcriptional start site, was efficiently transcribed in vivo (Fig. 6A) and yielded a transcript identical in size to that obtained from DZF1 (data not shown). Strains transduced with pLJS49-114 were as efficiently transcribed as strains transduced with pLJS49-111, which contained an additional 2 kb of DNA upstream of the *Hin*cII site shown in Fig. 6; however, neither construct was transcribed as efficiently as the parental strain DZF1 (data not shown). These results suggest the possible involvement of additional regulatory sequences which map upstream of the *M. xanthus* sequences contained in pLJS49-114 (Fig. 6A). Nevertheless, pLJS49-114 clearly contained at least one set of transcriptional signals capable of activating *mbhA* mRNA synthesis during development.

Plasmid pLJS49-114X1 contains a deletion of the σ^{54}-like sequence (nucleotides 334 to 349 in Fig. 4A) but is otherwise identical to pLJS49-114. Deletion of this sequence totally abolished promoter activity (Fig. 6B); developmental RNA extracted from strain DZF3608 yielded only the Tn*5*-truncated *mbhA* transcripts and appeared virtually identical in its Northern blot profile to RNA obtained from the parental strain DZF3598 (not shown), suggesting that the σ^{54}-like sequence is an essential part of the transcriptional signals of pLJS49-114. However, this sequence by itself was not sufficient for *mbhA* transcription, since a construct containing this sequence plus an additional 88 bp of upstream DNA was transcriptionally inactive (Fig. 6C), indicating that sequences upstream of the *Mlu*I site shown in Fig. 6 are important for the *mbhA* transcriptional process. A comparison of the levels of *mbhA* transcript in DZF3607 (Fig. 6A) and DZF3606 (Fig. 6C) indicates that these sequences must map between 89 and 276 nucleotides upstream of the σ^{54}-like sequence. Furthermore, the results presented in Fig. 6 indicate that these sequences must act in *cis*, since the merodiploid DZF3606 contains this region of DNA intact at its normal site on the chromosome. It should also be noted that similar amounts of Tn*5*-truncated transcripts were generated in each of the merodiploids, indicating that the absence of detectable full-length transcripts in DZF3606 and DZF3608 was not due to a general defect in developmental transcription in these strains.

Also shown, in Fig. 6D, is the Northern blot of developmental RNA from strain DZF3601 which contains a Tn*5-lac* insertion approximately 140 bp upstream of the σ^{54}-like sequence. In parallel to what was seen in the Western blot analysis of this strain (Fig. 5), the Tn*5-lac* insertion resulted in greatly reduced levels of *mbhA* transcription.

DISCUSSION

The results presented in this paper demonstrate that expression of the *mbhA* gene is controlled, at least in part, at the level of transcription. We have mapped the 5′ end of the *mbhA* mRNA (Fig. 3) and have found upstream sequences that display significant homology to the class of promoters recognized by the σ^{54} holoenzyme form of RNA polymerase in *E. coli* and other eubacteria (15, 23, 30; Fig. 4). Deletion of this putative promoter completely abolished *mbhA* transcription (Fig. 6). Although our data cannot rule out the possibility that an overlapping unidentified promoter sequence is involved, the fact that the σ^{54}-like sequence is

found at the correct distance upstream of the 5′ end of the mRNA, along with the similarity of this sequence to 64 known σ^{54} promoters, strongly suggests that the σ^{54}-like sequence functions as the promoter for *mbhA*. In addition, our results indicate that at least one region of DNA, located between 89 and 276 nucleotides upstream of the σ^{54}-like promoter, is necessary for the transcriptional process and that this region must be present in *cis*, since no *mbhA* transcriptional activity could be detected in a merodiploid strain in which these upstream sequences were deleted in *cis* but present in *trans* (Fig. 6C).

These observations are consistent with what is currently known about the transcription of σ^{54} holoenzyme-dependent genes; in all cases, the involvement of a transcriptional activator protein has been either directly demonstrated or inferred from the requirement for sequences upstream of the promoter that could serve as binding sites for an activator protein (23). In the case of the best-characterized σ^{54}-dependent gene, *glnA* of *E. coli* (which encodes glutamine synthetase), transcription involves the action of a transcriptional activator (NTRC), which is itself regulated by a protein kinase/phosphatase activity encoded by the *ntrB* locus. These two proteins define the sensor (NTRB) and effector (NTRC) functions of a two-component regulatory system which controls nitrogen utilization (1, 35).

It has been demonstrated, both in vivo (43) and in vitro (38), that the phosphorylated form of NTRC (NTRC-P) effects the isomerization of binary complexes between σ^{54} holoenzyme and the *glnA* promoter to transcriptionally active open complexes, a prerequisite step in the initiation of an RNA chain. Furthermore, NTRC shares certain characteristics of eucaryotic enhancer-binding proteins; although the NTRC binding region is normally located within 150 bp of the *glnA* promoter, movement of this region 1.4 kb upstream (39) or 0.95 kb downstream (34) had little effect on the ability of NTRC to activate transcription.

Our merodiploid studies (Fig. 6) have indicated a region of DNA upstream of the σ^{54}-like sequence where activation factors may bind. Very marked decreases in *mbhA* transcription were observed in three strains that contained transposon insertions in this region (Fig. 5). All three transposon insertions had similar effects on the levels of *mbhA* RNA observed by Northern blot hybridization (data not shown) and MBHA polypeptide synthesis observed by Western blot analysis (Fig. 5); transcription levels appeared to be reduced to similar extents but not abolished. We estimate the levels of *mbhA* mRNA in the Tn*5* insertion strains to be less than 5% of that found in wild-type DZF1 (41a). The 5′ end of the RNA synthesized in cells containing one of these insertions was identical to that of the wild type (Fig. 3, lane 3), suggesting that the *mbhA* transcript detected in these cells was accurately initiated from the *mbhA* promoter and was not due to transcriptional readout from the Tn*5* element.

One possible explanation for these observations is that all three of these transposons map between the σ^{54}-like sequence and the binding site for a putative upstream activator; the small amount of residual *mbhA* gene expression in the transposon-insertion strains might then be due to the increased distances (5.4 or 12 kb) between the promoter and activator binding sites, resulting in lower levels of activation. If this were the case, the binding site for the putative transcriptional activator would map to a region between 178 and 276 bp upstream of the σ^{54}-like sequence. An alternative explanation is that each of the transposons is inserted into an essential region of the *cis* element. Since there is no obvious sequence homology surrounding the insertion sites of all three transposons, it seems unlikely that all three insertions interfere with the binding for a single transcription factor, although this possibility is difficult to rigorously exclude. The transposons might also interfere with a binding site(s) for multiple factors which are required for optimal transcriptional efficiency. Such an arrangement is not unusual for eucaryotic promoters (3, 29, 44). This possibility is particularly intriguing since multiple transcription factors have been a proposed mechanism for tissue-specific gene expression in eucaryotic systems (44) and *M. xanthus* is a system for which a consideration of developmental fates is relevant. Clearly, additional studies are warranted to more precisely define the *cis*-acting sequences that we have initially characterized.

cis-acting upstream elements have also been implicated in the developmental regulation of the protein S genes of *M. xanthus* (17). These two genes, *ops* and *tps*, are tandemly arranged on the *M. xanthus* chromosome and are 90% identical at the DNA sequence level (18). Although they are differentially regulated (12, 47), both genes are transcriptionally activated by a segment of DNA which has enhancerlike properties (10, 20); additionally, expression of *ops* is dependent on the synthesis of a new RNA polymerase sigma factor, *sigB* (2). Thus, there are preliminary indications that developmental transcription in *M. xanthus* may involve relatively complex interactions of transcription factors.

σ^{54}-dependent genes have been identified in over 22 species of bacteria and have been found to be involved in such diverse functions as nitrogen regulation, flagellum biosynthesis, and amino acid transport (1, 32). In other systems in which σ^{54} holoenzyme promoters have been studied, it is frequently (but not always) observed that a two-component motif is involved in their regulation. Our data strongly suggest a requirement for a transcriptional activator, but there is as yet no evidence for another regulatory element contributing to *mbhA* transcription. However, since *mbhA* is efficiently expressed only at specific times of development, some form of developmental control for the synthesis or activation of the protein(s) regulating the expression of the *mbhA* promoter must obtain.

Genes that are transcribed by the σ^{54} holoenzyme often involve functions that are responsive to environmental stimuli. The nature of the stimulus which might mediate *mbhA* expression is not clear. One likely candidate is the necessity for growth on a solid substrate which is a precondition for *mbhA* expression (8, 12). A more intriguing possibility is suggested by recent experiments which show that MBHA polypeptide is detected only in cells which have not entered the aggregation center (37a). This finding suggests that the expression of *mbhA* may be not only temporally regulated but spatially regulated as well. These considerations indicate that *mbhA* should continue to be a valuable marker for the complex interactions that occur during the developmental program of *M. xanthus*.

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REFERENCES

1. **Albright, L. M., E. Huala, and F. M. Ausubel.** 1989. Procaryotic signal transduction mediated by sensor and regulator protein pairs. Annu. Rev. Genet. **23**:311–336.
2. **Apelian, D., and S. Inouye.** 1990. Development-specific sigma factor essential for late stage differentiation of *Myxococcus xanthus*. Genes Dev. **4**:1396–1403.
3. **Atchison, M. L.** 1988. Enhancers: mechanisms of action and cell specificity. Annu. Rev. Cell Biol. **4**:127–153.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
5. **Berg, D. E., A. Weiss, and L. Crossland.** 1980. Polarity of Tn*5* insertion mutants in *Escherichia coli*. J. Bacteriol. **142**:439–446.
6. **Campos, J. M., J. Geisselsoder, and D. R. Zusman.** 1978. Isolation of bacteriophage Mx4, a generalized transducing phage for *Myxococcus xanthus*. J. Mol. Biol. **119**:167–178.
7. **Cheng, Y., and D. Kaiser.** 1989. *dsg*, a gene required for cell-cell interaction early in *Myxococcus* development. J. Bacteriol. **171**:3719–3726.
8. **Cumsky, M., and D. R. Zusman.** 1979. Myxobacterial hemagglutinin: a development-specific lectin of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**:5505–5509.
9. **Cumsky, M. G., and D. R. Zusman.** 1981. Purification and characterization of myxobacterial hemagglutinin, a development-specific lectin of *Myxococcus xanthus*. J. Biol. Chem. **256**:12581–12588.
10. **Downard, J. S., S.-H. Kim, and K.-S. Kil.** 1988. Localization of the *cis*-acting regulatory DNA sequences of the *Myxococcus xanthus tps* and *ops* genes. J. Bacteriol. **170**:4931–4938.
11. **Downard, J. S., D. Kupfer, and D. R. Zusman.** 1984. Gene expression during development of *Myxococcus xanthus*: analysis of the genes for protein S. J. Mol. Biol. **175**:469–492.
12. **Downard, J. S., and D. R. Zusman.** 1985. Differential expression of protein S genes during *Myxococcus xanthus* development. J. Bacteriol. **161**:1146–1155.
13. **Garriga, G., H. Bertrand, and A. M. Lambowitz.** 1984. RNA splicing in *Neurospora* mitochondria: nuclear mutants defective in both splicing and 3′ end synthesis of the large rRNA. Cell **36**:623–634.
14. **Garriga, G., and A. M. Lambowitz.** 1984. RNA splicing in *Neurospora* mitochondria: self-splicing of a mitochondrial intron in vitro. Cell **38**:631–641.
15. **Gussin, G. N., C. W. Ronson, and F. M. Ausubel.** 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. **20**: 567–591.
16. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64**:284–296.
17. **Inouye, M., S. Inouye, and D. R. Zusman.** 1979. Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**:209–213.
18. **Inouye, S., Y. Ike, and M. Inouye.** 1983. Tandem repeat of the genes for protein S, a development-specific protein of *Myxococcus xanthus*. J. Biol. Chem. **258**:38–40.
19. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Annu. Rev. Genet. **20**:539–566.
20. **Kil, K.-S., G. L. Brown, and J. S. Downard.** 1990. A segment of *Myxococcus xanthus ops* DNA functions as an upstream activation site for *tps* gene transcription. J. Bacteriol. **172**:3081–3088.
21. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn*5-lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **81**:5816–5820.
22. **Kroos, L., and D. Kaiser.** 1987. Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. Genes Dev. **1**:840–854.
23. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of σ^{54} (NtrA)-dependent genes is probably united by a common mechanism. Microbiol. Rev. **53**:367–376.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
25. **LaRossa, R., J. Kuner, D. Hagen, C. Manoil, and D. Kaiser.** 1983. Developmental cell interactions of *Myxococcus xanthus*: analysis of mutants. J. Bacteriol. **153**:1394–1404.
26. **Maniatis, T., E. F. Fritch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Mead, D. A., E. Szczesna, and B. Kemper.** 1986. Single-stranded DNA "blue" T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Eng. **1**:67–74.
28. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
30. **Morett, E., and B. Martin.** 1989. *In vivo* studies of the interaction of RNA polymerase-σ^{54} with the *Klebsiella pneumoniae* and *Rhizobium meliloti nifH* promoters. J. Mol. Biol. **210**:65–77.
31. **Morrison, C. E., and D. R. Zusman.** 1979. *Myxococcus xanthus* mutants with temperature-sensitive, stage-specific defects: evidence for independent pathways in development. J. Bacteriol. **140**:1036–1042.
32. **Mullin, D., S. Minnich, L.-S. Chen, and A. Newton.** 1987. A set of positively regulated flagellar gene promoters in *Caulobacter crescentus* with sequence homology to the nif gene promoters of *Klebsiella pneumoniae*. J. Mol. Biol. **195**:939–943.
33. **Nelson, D. R., M. G. Cumsky, and D. R. Zusman.** 1981. Localization of myxobacterial hemagglutinin in the periplasmic space and on the cell surface of *Myxococcus xanthus* during developmental aggregation. J. Biol. Chem. **256**:12589–12595.
34. **Ninfa, A. J., L. J. Reitzer, and B. Magasanik.** 1987. Initiation of transcription at the bacterial *glnA*p2 promoter by purified components is facilitated by enhancers. Cell **50**:1039–1046.
35. **Nixon, B. T., C. W. Ronson, and F. M. Ausubel.** 1986. Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. Proc. Natl. Acad. Sci. USA **83**:7850–7854.
36. **Norander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene **26**:101–106.
37. **O'Connor, K. A., and D. R. Zusman.** 1983. Coliphage P1-mediated transduction of cloned DNA from *Escherichia coli* to *Myxococcus xanthus*: use for complementation and recombinational analyses. J. Bacteriol. **155**:317–329.

37a. **O'Connor, K. A., and D. K. Zusman.** Unpublished data.

38. **Popham, D., D. Szeto, J. Keener, and S. Kustu.** 1989. Function of a bacterial activator protein that binds to transcriptional enhancers. Science **243**:629–635.
39. **Reitzer, L. J., and B. Magasanik.** 1986. Transcription of *glnA* in *E. coli* is stimulated by activator bound to sites far from the promoter. Cell **45**:785–792.
40. **Romeo, J. M., B. Esmon, and D. R. Zusman.** 1986. Nucleotide sequence of the myxobacterial hemagglutinin gene contains four homologous domains. Proc. Natl. Acad. Sci. USA **83**:6332–6336.
41. **Romeo, J. M., and D. R. Zusman.** 1987. Cloning of the gene for myxobacterial hemagglutinin and isolation and analysis of structural gene mutations. J. Bacteriol. **169**:3801–3808.

41a. **Romeo, J. M., and D. R. Zusman.** Unpublished data.

42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
43. **Sasse-Dwight, S., and J. D. Gralla.** 1988. Probing the *Escherichia coli glnALG* upstream activation mechanism in vivo. Proc. Natl. Acad. Sci. USA **85**:8934–8938.
44. **Schule, R., M. Muller, C. Kaltschmidt, and R. Renkawitz.** 1988. Many transcription factors interact synergistically with steroid receptors. Science **242**:1418–1420.

45. **Shimkets, L. J.** 1987. Control of morphogenesis in myxobacteria. Crit. Rev. Microbiol. **14**:195–227.
46. **Shimkets, L. J., and S. J. Asher.** 1988. Use of recombination techniques to examine the structure of the *csg* locus of *Myxococcus xanthus*. Mol. Gen. Genet. **211**:63–71.
47. **Teintze, M., R. Thomas, T. Furuichi, M. Inouye, and S. Inouye.** 1985. Two homologous genes coding for spore-specific proteins are expressed at different times during development of *Myxococcus xanthus*. J. Bacteriol. **163**:121–125.
48. **Weinberg, R. A., and D. R. Zusman.** 1989. Evidence that the *Myxococcus xanthus frz* genes are developmentally regulated. J. Bacteriol. **171**:6174–6186.
49. **Zusman, D. R.** 1984. Cell-cell interactions and development in *Myxococcus xanthus*. Q. Rev. Biol. **59**:119–138.
50. **Zusman, D. R., M. G. Cumsky, D. R. Nelson, and J. M. Romeo.** 1986. Myxobacterial hemagglutinin: a developmentally induced lectin from *Myxococcus xanthus*, p. 197–216. *In* D. Mirelman (ed.), Microbial lectins and agglutinins. John Wiley & Sons, Inc., New York.