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Cloning of developmentally regulated flagellin genes from *Caulobacter crescentus* via immunoprecipitation of polyribosomes

(prokaryote/cDNA synthesis/DNA sequence/genetic analysis)

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ABSTRACT **Immunoprecipitation of *Caulobacter crescentus* polyribosomes with antiflagellin antibody provided RNA for the synthesis of cDNA probes that were used to identify three specific *Eco*RI restriction fragments (6.8, 10, and 22 kilobases) in genomic digests of *Caulobacter* DNA. The RNA was present only in polyribosomes isolated from a time interval in the *Caulobacter* cell cycle that was coincident with flagellin polypeptide synthesis. The structural gene for M_r 27,500 flagellin polypeptide was assigned to a region of the 10-kilobase *Eco*RI restriction fragment by DNA sequence analysis. Analysis of mutants defective in motility further established a correlation between the M_r 27,500 flagellin gene and the *flaE* gene locus [Johnson, R. C. & Ely, B. (1979) *J. Bacteriol.* 137, 627–634]. The other *Eco*RI fragments that hybridize with the immunoprecipitated polyribosome-derived cDNA probe are also temporally regulated and have features that suggest they encode other polypeptides associated with the flagellum. Modifications were required to adapt the procedure of immunoprecipitation of polyribosomes for use with *Caulobacter* and should be applicable to the production of specific structural gene probes from other prokaryotic systems.**

The molecular basis of selective gene expression and the spatial localization of cellular components are fundamental aspects of development and cell differentiation. Although interesting and important problems, a comprehensive molecular analysis of temporal and spatial control has not been possible. To gain an understanding of developmental regulation at this level we have studied the prokaryote *Caulobacter crescentus* (1). During the course of each cell cycle, these organisms undergo a well-defined series of morphological changes, producing, at each cell division, daughter cells that differ in morphology, biosynthesis capacity, and developmental fate.

The structural proteins of the *Caulobacter* flagellar apparatus are synthesized and assembled at a predetermined cell pole during a discrete interval of the cell cycle (1, 2). Several lines of evidence suggest that new transcription is required for the expression of the flagellar filament monomers (3, 4) and that there is a functional segregation of flagellin mRNA at each cell division.

With the isolation of appropriate gene probes, the entire flagellar system would provide an opportunity to examine both the regulation of gene expression and the segregation of informational molecules during development. Despite many and varied attempts, however, the flagellin genes remain elusive to isolation and identification by customary molecular genetic techniques. Furthermore, more than 27 genetic complementation groups have been defined in *Caulobacter*, which affect cell motility (5); however, none has been identified as flagellin structural gene loci.

In this context, we chose a different approach to prokaryotic gene isolation by using RNA derived from the specific immunoprecipitation of polyribosomes to provide a probe for flagellin genes. These studies notwithstanding, immunoprecipitation procedures have been successful only when applied to the isolation of genes from eukaryotic organisms and usually those producing significant quantities of a particular gene product. In this paper we report the successful adaptation of this method to the isolation of specific mRNA for *Caulobacter* flagellins which, at their maximal period of synthesis, have a mRNA half-life in the order of 4–6 min (2, 4) and account for ≈0.5% of total protein synthesized during a short period of the life cycle (6).

Three discrete *Eco*RI DNA fragments that hybridized with a polyribosome-derived cDNA probe were cloned into pBR325; one of these fragments was shown to contain the structural gene for the M_r 27,500 flagellin by DNA sequence analysis and by genetic criteria. The evidence suggests that the other DNA fragments also encode structural polypeptide(s) of the flagellar filament. Our studies have resulted in the isolation of a related, but unlinked, set of developmentally regulated prokaryotic structural genes. In addition, the method should be widely applicable for the isolation of structural genes in any prokaryotic system for which specific antibody is available.

MATERIALS AND METHODS

Cell Growth and Antibody Production. *C. crescentus* strain CB15 (ATCC 19089) and CB15 mutant SC520 defective in cell motility (5) were grown in minimal medium (HMG) (7). Synchronous populations of swarmer cells were obtained by the Ludox (Dupont; HS-40) technique (8). Monospecific antiflagellin antibody was prepared and characterized as described (9). Purified IgG was obtained by ammonium sulfate precipitation and DEAE-cellulose chromatography (10).

Production of RNase-Free Reagents. Elimination of RNase activity at all stages of the procedures described was crucial for success. Glassware and solutions were autoclaved under standard conditions. Equipment that could not be autoclaved was treated with diethylpyrocarbonate (Sigma) (11). Purified IgG antibody was depleted of RNase activity by repeated passage over 5′-(4-aminophenylphosporyl)uridine 2′-(3′)-phosphate-agarose (Miles) columns (12). Depletion of RNase activity was assessed by incubating aliquots of the purified antibody with RNA and then measuring RNA degradation by agarose gel electrophoresis. Fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem) were successively washed, pelleted, and resuspended in Na300 (50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/0.3 M NaCl)

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Abbreviation: kb, kilobase(s).

buffer containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate until no apparent RNase activity was detected; this usually required three to five washes.

Polyribosome Preparation and Immunoprecipitation. Polyribosomes were prepared essentially as described by Strome and Young (13) from 100-ml cultures of CB15 grown to an OD_{660} nm of 0.5–0.7, except that chloramphenicol (Sigma) was added to cultures prior to harvesting (10 μg/ml) and during lysis (50 μg/ml). Fractionated polyribosomes were pooled and collected by centrifugation at 100,000 × *g* for 5 hr in a Beckman type 65 rotor, resuspended in Na300 buffer, quick-frozen, and stored at −70°C.

Immunoprecipitation of polyribosomes was carried out as described by Gough and Adams (14). Pooled polyribosomes (17–30 $OD_{260\ nm}$ units; Fig. 1, fraction A) were incubated with RNase-free antiflagellin IgG and then with formalin-fixed *S. aureus* cells. The complex was centrifuged for 30 min at 12,000 × *g* at 4°C. To release total RNA from the complex, the pellet was suspended in Na300 buffer containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 20 mM EDTA and incubated at 4°C for 30 min. The *S. aureus* cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was then centrifuged for 3 hr at 100,000 × *g* to separate ribosomes from soluble RNA. The clear ribosomal pellet was discarded, the supernatant was extracted with phenol, and the aqueous layer was precipitated with ethanol. The resulting precipitate was used for single-stranded cDNA synthesis.

DNA Cloning. Genomic DNA was cut with *Eco*RI and fractionated by agarose gel electrophoresis. DNA fragments of appropriate size were excised from the gel and electroeluted in 50 mM Tris·HCl, pH 8.0/50 mM boric acid/1 mM EDTA at 80 V for 16 hr. The fragments were then purified by DEAE-cellulose chromatography and ethanol precipitation; purified fragments were ligated into the *Eco*RI restriction site of pBR325 which had been treated with bacterial alkaline phosphatase (Worthington). The ligation products were used to transform *Escherichia coli* C600 or HB101 to ampicillin resistance. The

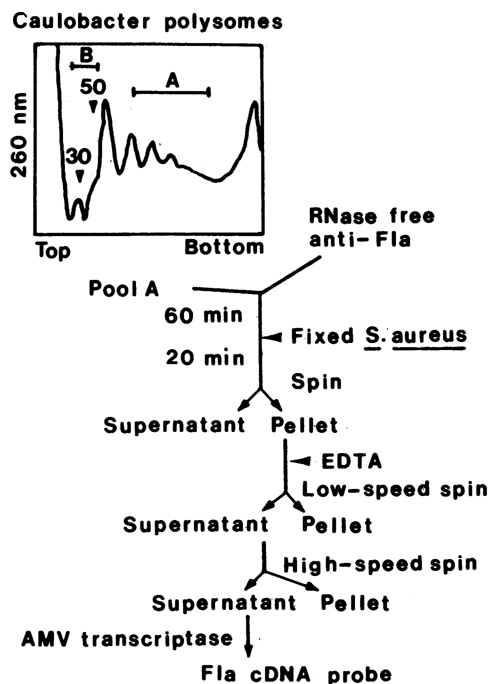

FIG. 1. Isolation and immunoprecipitation of polyribosomes and the subsequent production of cDNA probe. Fla, flagellin; AMV, avian myeloblastosis virus.

chloramphenicol-sensitive ampicillin-resistant colonies were screened by colony hybridization (15) using cDNA probe derived from immunoprecipitation of polyribosomes.

cDNA Synthesis, Nucleic Acid Isolation, Hybridization, and Electrophoresis. cDNA to the immunoprecipitated soluble RNA was synthesized by random priming using avian myeloblastosis virus reverse transcriptase (16). Chromosomal DNA was isolated from CB15 cultures according to the procedure of Marmur (17). Plasmid DNA was prepared according to the method of Bolivar *et al.* (18). DNA restrictions were carried out according to the manufacturer's (Bethesda Research Laboratories) specifications. DNA gels and nitrocellulose blotting were as described by Southern (19). Hybridizations were by the method of Thomashow *et al.* (20). $NaDodSO_4$ gel electrophoresis was as described (21). Protein blotting was by the method of Burnette (22), except that 3-chloro-4-naphthol (Sigma) was used as the chromophore for peroxidase localization.

DNA Sequence Analysis. Partial and complete digestions of the 2.2-kilobase (kb) *Sal* I fragment from pCA110 (see *Results*) were performed with a number of enzymes and cloned into the appropriate M13 cloning vehicles (23). These subclones were verified by hybridization to the 2.2-kb *Sal* I fragment. DNA sequence analysis of these subclones was by the chain termination method of Sanger and co-workers (24). The dideoxyribonucleotide triphosphates were obtained from PL Biochemicals. The M13 12-mer primer was obtained from Collaborative Research (Waltham, MA). The "large fragment" of *E. coli* DNA polymerase 1 was obtained from Boehringer Mannheim.

RESULTS

The cDNA probe, prepared from RNA derived from immunoprecipitated polyribosomes, hybridized uniquely with three *Eco*RI restriction fragments in genomic digests of *Caulobacter* DNA. The initial characterization, by Southern hybridization, of the DNA fragments as containing presumptive flagellin genes depended on two factors: (*i*) isolation of polyribosomes from cells during the period of flagellin polypeptide synthesis and (*ii*) inclusion of unlabeled rRNA as a competitor during hybridization.

The immunoprecipitation of polyribosomes with antibody to flagellin monomers is summarized in Fig. 1. After immunoprecipitation, the *S. aureus*–antibody–polyribosome complexes were disrupted by the addition of detergent and EDTA, and the majority of rRNA was separated, by high-speed centrifugation, as ribonucleoprotein complexes. The soluble RNA was extracted with phenol and concentrated by ethanol precipitation. This mRNA-enriched fraction was used to prepare single-stranded cDNA probes by random priming.

rRNA is a major contaminant in these preparations because of the inherent difficulty in achieving an effective separation of rRNA and mRNA from prokaryotes. Therefore, rRNA isolated from monosome peaks (Fig. 1, fraction B) was used to prepare a cDNA probe to identify the number and complexity of rDNA-containing fragments on restriction blots. Additionally, a cDNA probe prepared from RNA derived from polyribosomes immunoprecipitated with preimmune serum provided a control for nonspecifically precipitated mRNA.

The results of such preliminary immunoprecipitation experiments with preimmune serum, antiflagellin antibody, and isolated rRNA are shown in Fig. 2. The polyribosomes used in this experiment were isolated from asynchronous cultures. Lane 1 in Fig. 2A demonstrates those *Eco*RI DNA fragments that contained rDNA sequences. The identity of these DNA fragments as rDNA genes has been confirmed by subsequent cloning and restriction (unpublished data) and our data agree with the detailed map of rRNA genes recently published by Ohta and New-

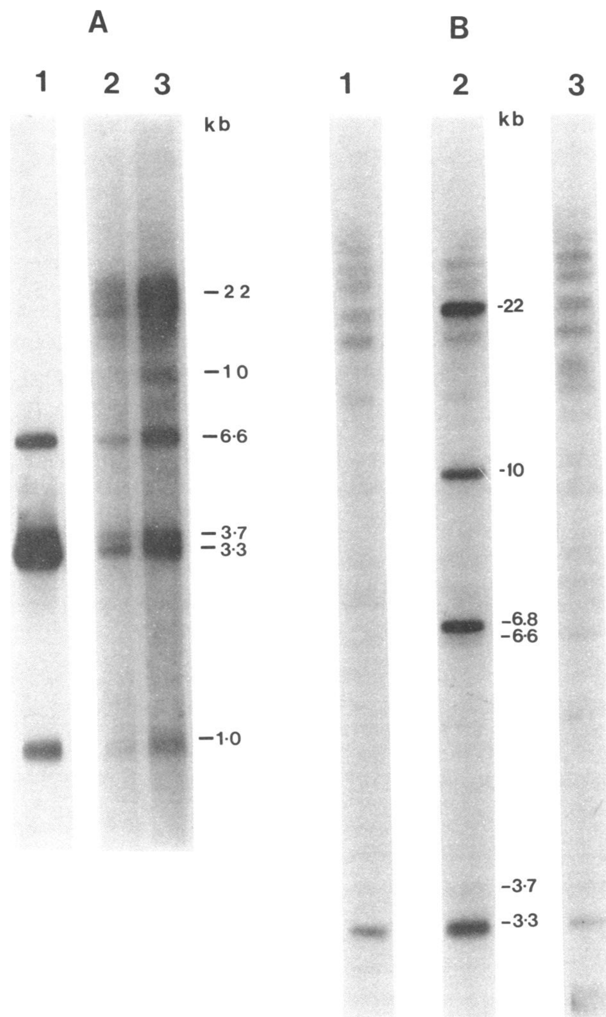

FIG. 2. Southern blot hybridization. *Caulobacter* DNA was digested with *Eco*RI, fractionated on a 0.7% agarose gel, and transferred to a nitrocellulose sheet by the method of Southern (19). Each sample (3 μg of DNA) was hybridized with ^{32}P-labeled cDNA probes (3×10^6 cpm) prepared as indicated. (*A*) Lane 1, cDNA was prepared from isolated rRNA. Lanes 2 and 3, cDNA was prepared from RNA derived from polyribosomes which were precipitated with preimmune serum (lane 2) or antiflagellin antibody (lane 3). In both cases, asynchronous cell populations were used. (*B*) cDNA was prepared from RNA derived from polyribosomes isolated at various stages in the cell cycle. Each hybridization mixture contained an additional 50 μg of unlabeled rRNA. Lanes: 1, stalked cell polyribosomes precipitated with antiflagellin antibody; 2, predivisional cell polyribosomes precipitated with antiflagellin antibody; 3, predivisional cell polyribosomes precipitated with preimmune serum.

ton (25). Lane 2 represents the DNA fragments hybridizing with background levels obtained by precipitation of polyribosomes with preimmune serum. rRNA is again the major contaminant although several minor hybridizing species were also detected. Lane 3 represents those fragments that hybridize with the cDNA probe derived from the specific immunoprecipitation of polyribosomes with antiflagellin antibody. In this hybridization, rRNA and RNA species present in the preimmune control predominate, but there is a clear indication of additional *Eco*RI fragments that hybridize to 10-kb, and perhaps to 6- and 22-kb, regions of the genomic digest.

Because flagellin polypeptides are synthesized only in swarmer and predivisional cell stages, polyribosomes actively synthesizing flagellin should not be present in stalked cells. As an additional criterion of specificity of immunoprecipitation, therefore, polyribosomes were isolated from synchronous stalked and predivisional cell populations and used as a source of mRNA for preparation of the cDNA probe. In addition, unlabeled rRNA, at 50 μg/ml in each hybridization reaction, was added to compete with the hybridization contribution of labeled rRNA in the cDNA probe. Fig. 2*B* represents the hybridization profile of genomic DNA cut with *Eco*RI and hybridized to the cDNA probes derived either from polyribosomes precipitated with preimmune serum or polyribosomes isolated from predivisional cells and stalked cells precipitated with antiflagellin IgG. The pattern of cDNA hybridization obtained from stalked cell RNA derived probes does not differ significantly from that obtained with cDNA prepared from polyribosomes precipitated with preimmune serum. The resolution of the three presumptive flagellin genes containing DNA fragments in Southern blots was enhanced by competition with unlabeled rRNA. In the case of the 6.8- and 22-kb fragments, this was essential to their identification. By adequately controlling for nonspecific mRNA precipitation with preimmune serum, as well as identification and suppression of rRNA hybridization, it was possible to identify hybridization with cDNA probes obtained by immunoprecipitation of polyribosomes with antiflagellin antibody. The appearance of the three *Eco*RI fragments at 6.8, 10, and 22 kb is dependent upon immunoprecipitation of polyribosomes with specific antiflagellin antibody during the interval of the cell cycle when flagellin polypeptides are synthesized.

*Eco*RI restriction fragments of approximately 6.8, 10, and 22 kb were cloned and screened by colony hybridization with a cDNA probe similar to that shown in lane 2 of Fig. 2*B*. cDNA probes used for screening putative clones were hybridized with genomic DNA *Eco*RI digests to ensure purity of the probe. Clones of the 6.8- and 10-kb *Eco*RI fragments were obtained; perhaps due to its size, the 22-kb clone was not stable as such in *E. coli*. Clones containing either 6.8- or 10-kb DNA fragments were mapped with restriction endonucleases, and the regions complementary to the cDNA probe were determined by hybridization (data not shown). In each case the cDNA probe hybridized to a single contiguous region of the cloned insert (Fig. 3). The region of pCA160 that hybridized with the cDNA probe is within an 0.9-kb *Eco*RI/*Sal* I and a 1.1-kb *Sal* I fragment; that in pCA110 is within a 2.2-kb *Sal* I fragment.

The DNA fragments that hybridized with the immunoprecipitated polyribosome-derived probe were highly reproducible and dependent upon antiflagellin antibody precipitation during restricted periods of the cell cycle. However, these clones did not produce *Caulobacter*-specific polypeptides in *E. coli* maxi cells (26) or in chloramphenicol-release experiments

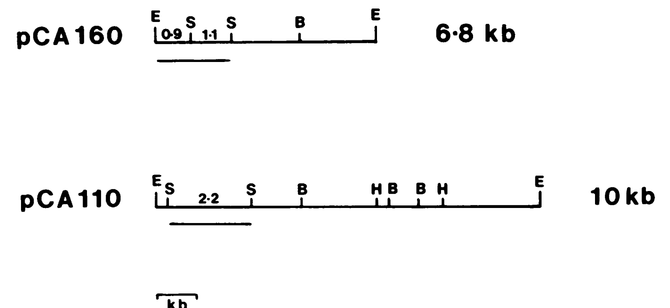

FIG. 3. Restriction map of pCA110 and pCA160. The solid line beneath each clone represents the region of hybridization with the specific cDNA probe produced from immunoprecipitation of polyribosomes with antiflagellin antibody in the 10- and 6.8-kb *Eco*RI DNA restriction fragments, respectively. The numbers within this region identify the sizes of the fragments in kb. E, *Eco*RI; S, *Sal* I; B, *Bam*HI; H, *Hin*dIII.

(27). Although the stage-specific immunoprecipitation of the hybridizing sequences provided strong presumptive evidence that these fragments encoded flagellins, we sought additional criteria in their identification.

Because we had previously established the NH_2-terminal amino acid sequence of the two major flagellin subunits (28), the regions in clones pCA160 and pCA110 that hybridized with polyribosome-derived probe were subcloned into M13 for DNA sequence analysis. A 42-nucleotide region approximately in the center of the 2.2-kb *Sal* I fragment from pCA110 had a sequence in complete agreement with the available protein sequence for M_r 27,500 flagellin and sufficient to distinguish it from the M_r 25,000 flagellin subunit (Fig. 4). The sequence of the structural gene, its flanking regions, and its relationship to the other polyribosome-derived clones will be described in detail elsewhere.

Twenty-seven complementation groups that affect *Caulobacter* motility and that are dispersed in at least five unlinked regions in the genome have been reported (5). With the structural gene of the M_r 27,500 flagellin identified, the genetic map location was correlated with the physical map by using subcloned regions of pCA110 as a probe. DNA was isolated from more than 70 different mutants generated by chemical or spontaneous mutagenesis or by Tn5-mediated transpositional inactivation of the motility phenotype. All of the mutants were analyzed both by Southern hybridization with pCA110 and protein blotting of whole-cell cultures with antiflagellin antibody. One mutant, SC520, which had been described by Johnson and Ely as a *flaE* mutant (5), illustrates the relationship between *flaE* and M_r 27,500 flagellin (Fig. 5). Mutant SC520 results from the deletion of approximately 1.7 kb within pCA110. It has lost the ability to synthesize M_r 27,500 flagellin. Analysis with subcloned regions of pCA110 demonstrated that the deletion maps within the M_r 27,500 flagellin structural gene. The deletion of a region that hybridized with the cDNA probe and the consequent loss of the flagellin gene sequence provides an additional criterion for the specificity for the immunoprecipitation of polyribosomes with antiflagellin antibody.

DISCUSSION

Caulobacter flagellin gene sequences have been cloned after identification of DNA fragments in Southern blots with a cDNA probe derived from polyribosome-associated mRNA, enriched by immunoprecipitation with specific antibody. The isolation and identification of the M_r 27,500 flagellin gene has been verified directly by DNA sequence and analysis of deletion mutants. Although a thorough analysis has not been completed with pCA160 and the 22-kb *Eco*RI restriction fragment, we are confident that these are also related to flagellin structural genes. In addition to being expressed in a stage-specific fashion and their products being specifically immunoprecipitated by antiflagellin antibody (this paper), both pCA160 and the 22-kb *Eco*RI fragment are homologous with the M_r 27,500 structural gene probe as determined by Southern hybridization (unpub-

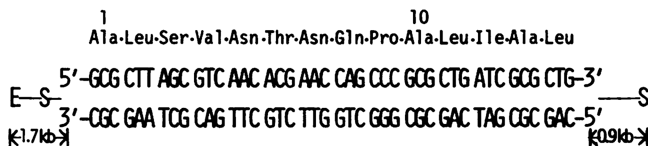

FIG. 4. NH_2-terminal amino acid sequence of the M_r 27,500 flagellin monomer and the corresponding segment of the flagellin structural gene, showing thé orientation and approximate location of the 42-base-pair sequence with respect to the *Eco*RI (E) and *Sal* I (S) restriction sites of pCA110 as shown in Fig. 3. The amino acids are numbered from the NH_2 terminus of the mature protein (28).

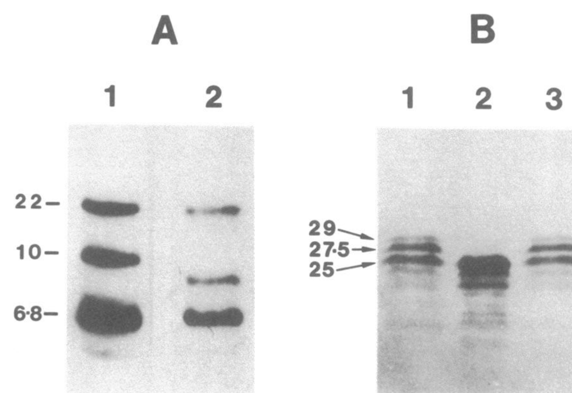

FIG. 5. Southern hybridization and protein blot analysis of CB15 and SC520. (*A*) Southern blots of *Caulobacter* DNA digested with *Eco*RI were prepared as described in Fig. 2. ^{32}P-Labeled M13 derived probe (7×10^6 cpm) from pCA160 hybridized to each of the *Eco*RI fragments (22, 10, and 6.8 kb) identified in Fig. 2*B*. Lanes: 1, CB15 wild-type DNA (1.5 μg); 2, mutant SC520 (0.5 μg). (*B*) Protein blot analysis of whole-cell extracts from lanes 1 and 2. After electrophoretic transfer to nitrocellulose, flagellin polypeptides were visualized by using antiflagellin antibody.

lished data). Because flagellin polypeptides of M_r 29,000, 27,500, and 25,000 all are immunologically related (6) and the latter two are homologous at the protein sequence level (28, 29), it is possible that each of the hybridizing regions could code for a flagellin polypeptide(s). These three hybridizing regions have been mapped and found to be unique (unpublished data). Thus, immunoprecipitation of polyribosomes with antiflagellin antibody results in the unequivocal identification of one of the flagellin polypeptide encoding regions and probably reflects the arrangement of an unlinked family of genes related to flagellin polypeptide synthesis.

The isolation of prokaryotic genes, especially those under complex regulatory control, may not be generally feasible by screening for polypeptide expression in heterologous systems. Numerous efforts to identify flagellin genes by analyzing the host vector for polypeptide production from cloned DNA fragments have been unsuccessful. Recent work has demonstrated expression of some *Caulobacter* polypeptides in heterologous systems—namely, some tryptophan biosynthetic enzymes (M. Winkler, personal communication) and the *Caulobacter* hook polypeptide (30) in *E. coli*. In neither case is there any direct evidence that this occurs by recognition of *Caulobacter* promoters in the *E. coli* host vector. Further indications that uniform recognition of *Caulobacter* promoters in heterologous systems is not likely to occur comes from work by K. Amemiya and L. Shapiro (personal communication) who have found that, *in vitro*, *E. coli* RNA polymerase does not recognize some of the early phage promoters of the *Caulobacter* phage ϕCd1. The pCA110 flagellin clone described in this paper also does not express *Caulobacter* flagellin polypeptide in *E. coli*.

The technique of precipitating polyribosomes to isolate specific mRNA has been used in many eukaryotic systems (see ref. 14), notably those in which the mRNA represents a major proportion of the total mRNA. This technique, however, has not been used successfully to isolate a specific mRNA from prokaryotic organisms. It is feasible in eukaryotes because eukaryotic mRNA is more stable than prokaryotic mRNA and can be functionally separated from other RNAs by virtue of its poly(A) tracts; nonspecific precipitation of mRNAs remains the single most persistent problem in the application of these procedures to gene isolation. In adapting the immunoprecipitation procedures to prokaryotic polyribosomes, several specific measures

were required. Nonspecific immunoprecipitation was minimized or accounted for by using *S. aureus* cells to effect precipitation and by assessing the contribution of minor RNA species by deriving a probe from preimmune serum precipitation reactions. It was also essential to determine the location of rDNA in appropriate restriction digests and to suppress the hybridization of cDNA derived from rRNA by competitive hybridization. Immunoprecipitation of polyribosomes from *Caulobacter* cell populations not expressing flagellin provided an additional control for nonspecific immunoprecipitation and fulfilled the criterion of stage-specific expression. We believe that the method outlined above can be applied to the isolation, from prokaryotes, of various structural genes for which monospecific antibody is available.

The regulation of prokaryotic development is generally patterned on models of bacteriophage infection (31, 32), sporulation (33, 34), and fruiting body formation (35) in which differentiation is essentially unidirectional. Modification of the host- or stage-specific RNA polymerases and the consequent changes in transcriptional specificity reflect a commitment of the organism to an alternative differentiation pathway. In *Caulobacter*, however, changing transcriptional specificities represent a periodic clock which is repeatedly coordinated with the cell cycle. Additionally, the mechanism of achieving transcriptional selectivity is somehow partitioned between daughter cells because each has distinct patterns of gene expression (2). The isolation of specific *Caulobacter* genes encoding polypeptides that are both temporally controlled and spatially determined is an important prerequisite in the molecular analysis of gene expression in this system. Using the cloned flagellin gene for example, we have shown directly that the stage-specific biosynthesis of flagellin is regulated at the level of transcription as previously suggested by studies with the drug rifampicin (3). With respect to problems of spatial determination, functional segregation of flagellin mRNA, which had been proposed as a result of a number of biological experiments (2, 4, 6), can be directly assayed with specific flagellin gene probes. By hybridization of cloned genes with RNA isolated from sibling stalked and swarmer cells and from the parent cell prior to cell division we have found that *Caulobacter* flagellin mRNA segregates specifically with the swarmer cell at the time of cell division (unpublished data).

From the beginning of experimental embryology, the study of development has been concerned with mechanisms by which cells generate and maintain structural and functional differences. Studies in recent years have shown that asymmetry in *Caulobacter* cells is reflected not only in the morphology of each of the progeny cells but also is extended to differential patterns of protein synthesis (36), chromosome structure (8), chromosomal protein (37), membrane proteins (2), and organelle biosynthesis (9, 38, 39, 40). With the isolation of specific genes that manifest these features of temporal and spatial regulation, the molecular basis of these events is now accessible to further analysis.

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