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Isolation and expression of cloned hook protein gene from *Caulobacter crescentus*

(cloning/peptide mapping of hook protein)

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ABSTRACT **Previous genetic analysis of *Caulobacter crescentus* showed that the periodic synthesis of hook protein, flagellin A, and flagellin B, the major flagellar subunits, is coupled in some way to chromosome replication. To examine the regulation of flagellar gene expression at the molecular level, we isolated the gene that codes for the 72,000-dalton hook protein. A specific ^{125}I-labeled anti-hook protein IgG was used to screen a hybrid λL47.1 bank of 4,500 clones and to compare peptide maps of the cloned gene product with purified hook protein. Restriction analysis of DNA from the positive λ clones and plasmid subclones showed that the structural gene for the hook protein is contained on a 2.3-kilobase (kb) *Bam*HI fragment. The direction of transcription was established by demonstrating the inducibility of hook protein gene in strains with the 2.3-kb fragment fused to the *Escherichia coli* lipoprotein gene–lactose gene promoter-operator region of pIN-II. Preliminary genomic analysis showed that the hook gene occupies a single location on the *C. crescentus* chromosome. These results suggest that the periodic expression of the hook protein gene in the cell cycle does not involve a major or persistent rearrangement of the 2.3-kb coding sequence during the cell cycle.**

The extent to which differential gene expression is responsible for the generation of new cell types is a recurring question in developmental biology. It is possible to address this problem most directly in simple systems in which the new cell types and their differentiated products can be easily isolated and studied. One such system is the prokaryote *Caulobacter crescentus*. This aquatic bacterium has an invariant life cycle in which a stalked cell divides repeatedly to produce new swarmer cells with a well-defined set of polar structures, including the flagellum, pili, and DNA phage receptor sites. These structures are assembled in discrete stages during the stalked cell cycle and they segregate uniquely with the motile swarmer cell at division (for review of cell cycle, see refs. 1 and 2).

Much of the work on developmental regulation in *C. crescentus* has centered on flagellum biosynthesis, mainly because of the ease with which this structure can be isolated and the unusual role of the cell cycle in its formation. The three major subunits of the flagellum—hook protein, flagellin A, and flagellin B—are synthesized periodically in the cell cycle at times that coincide with hook and filament assembly (3–6). Furthermore, analysis of conditional cell cycle mutants suggests that a specific stage of chromosome replication is required for the onset of synthesis of flagellin (4) and hook protein (6). Regulation of flagellar genes probably occurs at the transcriptional level because their induction, as well as other developmental changes (7), requires *de novo* RNA synthesis (4, 6).

C. crescentus is unusual in that the synthesis of a relatively large number of proteins is modulated during the cell cycle (8–10), but the function of most of these proteins is unknown. In addition, we find that few of the periodically synthesized proteins are under cell cycle control (unpublished data). As a consequence, the flagellar genes at present are the most useful DNA sequences for the study of polar development and differential gene expression in these cells.

We report in this communication the isolation and preliminary characterization of the structural gene for the hook protein of *C. crescentus*. This gene was identified and the product of the cloned gene was characterized by a combination of immunologic techniques that should be generally applicable when homologous RNA or DNA probes are not available.

MATERIALS AND METHODS

Bacterial and Phage Strains. *C. crescentus* strains used were the wild-type strain CB15 (ATCC 19089) and the polyhook mutant PCM103 which overproduces hook protein (11). *Escherichia coli* strain Q358 was used to propagate λL47.1 (12) and hybrid λL47.1. The φP2 lysogen of strain Q358, Q359, was used to select and screen hybrid λL47.1 particles with *C. crescentus* DNA (CB15 DNA) inserts as described (12). Strains Q358 and Q359 were originally obtained from W. J. Brammer. *E. coli* strain C600(λcI857) was the host for transformation by pBR322 DNA (13), and strain C600(λcI857)/F'*lac* i^Q *pro*$^+$ was the host for transformation by pIN-II DNA (14).

Enzymes. Restriction nucleases were obtained from Bethesda Research Laboratories and T4 DNA ligase and DNA polymerase I were a gift from P. Schedl. δ-Chymotrypsin was obtained from Worthington; Pronase was from Calbiochem.

DNA Purification and Construction of λL47.1 Clone Bank. CB15 DNA was purified as described (15) from exponentially growing cells cultured in peptone-yeast extract medium (1). DNA fragments for cloning were prepared by partial digestion with serially diluted *Sau*3A and fractionation of the products by sucrose density gradient centrifugation (15). Fragments of 5–20 kilobases (kb) were collected and inserted in the *Bam*HI site of λL47.1 (12). After ligation the DNA was packaged *in vitro* (16), and isolated plaques were picked and amplified. Hybrid plaques were stored at −18°C in 50% glycerol (15). Plasmids pBR322 and pIN-II were prepared according to Clewell and Helinski (17).

Hook Protein Screen. λL47.1 and plasmid clones were screened for the production of hook protein by using ^{125}I-labeled rabbit anti-hook protein IgG (11) in the sandwich radioimmunoassay developed by Broome and Gilbert (18).

Characterization of Hook Protein Gene Product. Proteins produced by hybrid λL47.1 phages were labeled by infecting

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Abbreviations: kb, kilobase(s); CB15 DNA, *C. crescentus* DNA; Tc, tetracycline; Ap, ampicillin; *lpp*, lipoprotein gene; *lac*, lactose gene; kDal, kilodalton.

UV-irradiated *E. coli* strain 159 in the presence of [^{35}S]methionine as described by Jaskunas *et al.* (19). Hook protein in cell extracts was identified by radioimmunoassay (6). Hook protein produced by recombinant plasmids was further analyzed as follows. The antigen was first precipitated from total unlabeled cell extracts with anti-hook protein antiserum and electrophoresed on a 10% $NaDodSO_4$/polyacrylamide gel (20). Bands corresponding to the position of the hook protein on the gel were cut out and subjected to partial proteolysis *in situ* (21), and the products were separated by electrophoresis on a 13% $NaDodSO_4$/polyacrylamide gel. Polypeptides were transferred to nitrocellulose filters (Schleicher & Schuell B85) by electrophoresis for 16–20 hr at 4°C (22). The protein blots were soaked for at least 30 min at 37°C in 10 mM Tris, pH 7.4/saline that contained 10% preimmune rabbit serum and 3% bovine serum albumin. The filters were then incubated with ^{125}I-labeled anti-hook protein IgG in the same buffer for 1.5 hr at room temperature, washed according to Burnette (22), and autoradiographed.

RESULTS

λL47.1 Library and Radioimmunologic Screen. Because neither homologous mRNA nor complementation tests were available for identification of *C. crescentus* genes, an immunological procedure described by Broome and Gilbert (18) was used to screen a CB15 genomic library in λL47.1. This sandwich screen depends on the transcription and translation of the desired gene in *E. coli* to produce an antigen that will react first with a purified antibody attached to a solid plastic matrix and then with the same antibody labeled with ^{125}I. When anti-hook protein IgG was used in the assay, the response in screening hybrid λL47.1 clones was somewhat variable (see *Discussion*), but the assay was very sensitive with a lower limit of detection ranging from 0.05 to 0.1 ng of hook protein (Fig. 1A). When antiflagellin IgG was used, a minimum of ≈0.1 ng of flagellin could be detected (data not shown).

Because of the high G+C content of CB15 DNA (1), partial *Sau*3A digests of genomic DNA were used in an effort to construct a random library of *C. crescentus* genes. The restriction fragments were sized and inserted in the *Bam*HI site of λL47.1 (12). Approximately 4,500 independent hybrid λL47.1 clones were transferred to microtiter dishes, printed on lawns of the *E. coli* host, and screened in parallel with two antibodies—one that reacted specifically with hook protein and one that reacted with both flagellin A and flagellin B. Four clones [λL47.1Cc8855, λL47.1Cc8861, λL47.1Cc10637, and λL47.1Cc11018 (Fig. 1A)] were detected in the hook protein screen, but none was detected in the flagellin screen. Because the genome size reported for *C. crescentus* is about that of *E. coli* and the average λL47.1 insert was about 10 kb, the probability of finding a given gene in 2,300 clones is 99% if the *Sau*3A fragments were generated at random. Thus, as considered below (*Discussion*), the failure to identify a flagellin clone in the λL47.1 bank is unexpected.

Radioimmunoassay for Cloned Gene Product. Products of the λL47.1Cc11018 clone were examined by infecting UV-irradiated *E. coli* cells with the hybrid bacteriophage and labeling with [^{35}S]methionine. Radioimmunoassay of the cell extracts for hook protein showed that the clone directed the incorporation of ^{35}S-label into a 72-kilodalton (kDal) protein (Fig. 2); this labeled protein was not detected in extracts of cells infected by the λL47.1 parent vector, in extracts of uninfected cells (data not shown), or when nonimmune serum was used in the assay. The ability of purified hook protein to compete with the labeled 72-kDal protein in the radioimmunoassay confirmed the conclusion that the hook protein and the protein expressed by the CB15 DNA insert have common antigenic sites. Essentially the same results were obtained with λL47.1Cc8861 (data not shown). A more detailed characterization of the antigen is outlined below.

Restriction Maps of λL47.1 Hybrid Clones. Restriction analysis of DNA from the four positive λL47.1 hybrid phages showed that the cloned inserts contain an overlapping set of sequences from the same region of the *C. crescentus* genome, with a 5-kb *Sau*3A–*Sau*3A fragment common to all CB15 inserts (Fig. 3). The only exception to this arrangement is the leftmost segments of the λL47.1 Cc8855 and λL47.1Cc10637 inserts. When Southern blots (24) of complete *Bam*HI digests of genomic DNA were probed with the nick-translated (25) λL47.1Cc8855 DNA, the 7-kb *Bam*HI fragment of the Cc8855 insert was detected but the 5.8-kb fragment of the Cc10637 insert was not. Similarly, the 8.4-kb *Hin*dIII fragment of Cc8855

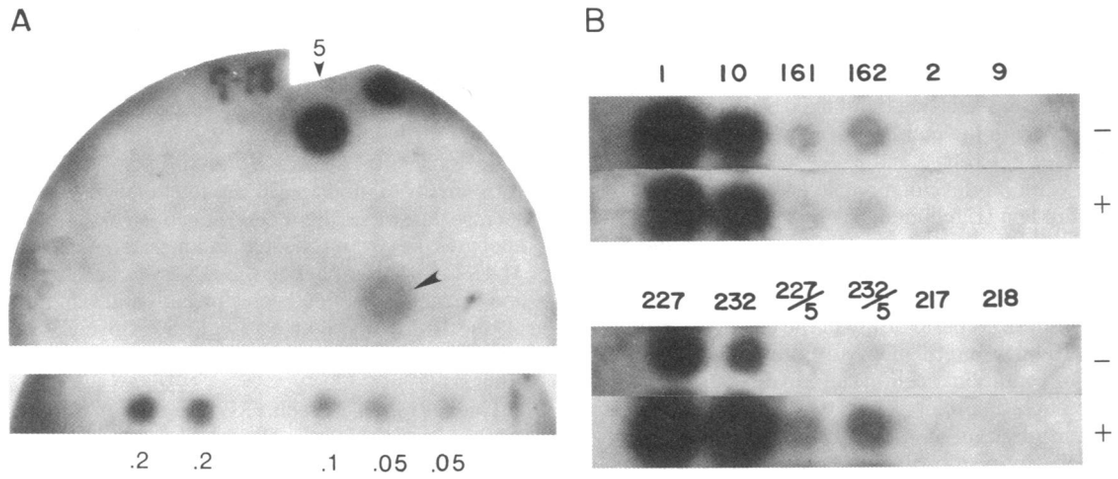

FIG. 1. Radioimmunologic sandwich screen for hook protein. (*A*) Screen of hybrid λL47.1 bacteriophages. Phages stored in microtiter wells were printed on a lawn of *E. coli* Q358 on LB agar plates (23), incubated overnight at 37°C, and screened. Only 1 of the 24 hybrid phage shown, λL47.1Cc11018 (arrowhead), gave a positive response. Hook protein at 0.05 to 5 ng was spotted on a lawn of the same strain as indicated. (*B*) Inducibility of hook protein gene fused to *lac* promoter-operator. λcI857 lysogens of *E. coli* containing pBR322 (*Upper pair*) or pIN-II (*Lower pair*) (see Table 1) were printed from microtiter dishes to LB agar plates without and with (2 mM) isopropyl thiogalactoside (− and +, respectively). After overnight incubation at 30°C the colonies were lysed by incubation at 42°C for 2 hr and assayed as in *A*. Hybrid phage designated 227/5 and 232/5 were printed from wells that contained the two subclones diluted 4:1 with the pIN-II parent vector.

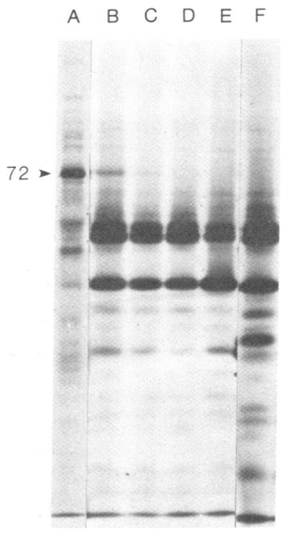

FIG. 2. Synthesis of hook protein by a hybrid λL47.1 clone in UV-irradiated *E. coli* cells. Irradiated *E. coli* strain 159 was infected with hybrid λL47.1Cc11018 (lanes B–E) or λL47.1 (lane F) and labeled with [^{35}S]methionine. *C. crescentus* strain PCM103 cells, which overproduce hook protein (11), were labeled as a control in the same experiment (lane A). All cell extracts were assayed for hook protein synthesis by using rabbit hook protein antiserum as described (11), except for one sample in which nonimmune rabbit serum was substituted (lane E). Purified hook protein at 20 μg (lane C) and 60 μg (lane D) was added to two samples before the radioimmunoassay. The heavy background in lanes B–F is characteristic of radioimmunoprecipitates from *E. coli* cell extracts with *C. crescentus* anti-hook protein antibodies. The 72-kDal position is indicated.

was detected but the 6.4-kb fragment of Cc10637 was not present in the genomic digestion (data not shown). Therefore, part of the λL47.1Cc10637 insert must have arisen from a rearrangement of genomic sequences during cloning. With this exception, the *Bam*HI, *Hin*dIII, and *Eco*RI restriction patterns of the λL47.1 hybrid inserts and of the homologous chromosomal region are identical (Fig. 3). Thus, the DNA sequence containing the hook protein gene (see next section) apparently occupies a unique position on the *C. crescentus* chromosome. These preliminary results are important in considering how the periodic expression of the hook protein gene may be regulated because they do not indicate a major or persistent rearrangement of the sequences during the cell cycle (see *Discussion*).

Characterization of pBR322 Subclones. To define the 72-kDal protein coding sequence further, DNA from λL47.1Cc8861 and λL47.1Cc11018 was partially cleaved with *Bam*HI and cloned into the corresponding restriction site of pBR322. Ninety-two ampicillin-resistant, tetracycline-sensitive (Ap^R,Tc^S) transformants of *E. coli* C600 (λcI857) were screened with the sandwich assay; 30 of these subclones were strongly positive, 4 were weakly positive, and the remainder were negative (Fig. 1*B*). Each of the 15 strongly positive subclones analyzed (e.g., S1 and S10) contained only the 2.3-kb *Bam*HI fragment, whereas the two weakly positive subclones S161 and S162 contained the 2.3-kb fragment plus the 0.67-kb *Bam*HI fragment (Table 1; Fig. 3). All *Bam*HI inserts in positive subclones were integrated in the same orientation relative to the Tc promoter of pBR322. Most of the negative pBR322 subclones examined contained either no CB15 DNA insert [e.g., S2 (Fig. 1*B*), or the 2.3-kb *Bam*HI fragment in the opposite orientation (e.g., S6 and S9) (Table 1)].

These results indicate that the gene for the 72-kDal protein is within the 2.3-kb fragment and that the pBR322 Tc promoter may be required for efficient expression of the sequence in *E. coli*. Consistent with the latter conclusion is the observation that subclones with the 4.2-kb *Hin*dIII fragment of Cc10637 inserted in the *Hin*dIII site of pBR322, which is upstream from the Tc promoter (26), do not produce a detectable 72-kDal protein (data not shown).

Direction of Transcription. The 2.3-kb *Bam*HI fragment was also subcloned in the pIN-II vector described by Inouye *et al.* (14). The *Bam*HI site in this plasmid is adjacent to the fused *E. coli* lipoprotein (*lpp*) promoter and *lac*UV5 promoter-operator sequences so that transcription of inserted DNA is initiated at a high level from the *lpp* and *lac* promoters when a *lac* inducer is present. *Bam*HI fragments from complete digests of λL47.1Cc8861 DNA were ligated to a mixture of pIN-II DNAs with the *Bam*HI sites in the three possible reading frames (14). Ap^R transformants of *E. coli* C600(λcI857) grown in the presence and absence of isopropyl β-D-thiogalactoside were screened by using the sandwich assay for hook protein. All of the positive pIN-II subclones (e.g., S227 and S232) were inducible (Fig. 1*B*) and they contained the 2.3-kb fragment of CB15 DNA in the same orientation; the pIN-II subclones that failed to produce hook protein (e.g., S217 and S218) contained the 2.3-kb fragment in the opposite orientation (Table 1). Comparison of the plasmid subclones that produced hook protein showed that the 2.3-kb fragments were integrated in the same orientation relative to the *lpp–lac* promoters in pIN-II and to the Tc promoter in pBR322 (Fig. 3). These results then established the direction of transcription of the gene (Fig. 3), and fusion of the sequence to the *lpp* promoter provided adequate amounts of the 72-kDal product for use in peptide mapping experiments.

Peptide Mapping of Cloned Hook Protein Gene Product. Because identification of the hook protein gene relies on the identity of the 72-kDal protein produced by the cloned CB15 DNA fragment in *E. coli* cells with the hook protein of *C. cres-*

Table 1. Plasmid subclones containing 2.3-kb *Bam*HI fragment

Subclone	λL47.1 parent	Insertion site	Insert, kb	Orientation*	Expression†
pBR322·S1	Cc11018	*Bam*HI	2.3	←	++
pBR322·S10	Cc 8861	*Bam*HI	2.3	←	++
pBR322·S161,S162	Cc11018	*Bam*HI	2.3 + 0.67	←	+
pBR322·S6, S9	Cc11018	*Bam*HI	2.3	→	−
pBR322·S21,S179	Cc10637	*Hin*dIII	6.2 + 4.2	ND	−
pIN-II·S227,S232	Cc 8861	*Bam*HI	2.3	←	+++
pIN-II·S217,S218	Cc 8861	*Bam*HI	2.3	→	−

* Orientation of 2.3-kb *Bam*HI fragment: ←, insertion of fragment allowing transcription from Tc or *lac–lpp* promoter in the direction indicated in Fig. 3; →, insertion of fragment in opposite orientation; ND, not determined.

† Level of hook protein expression estimated from sandwich assay (see Fig. 1). −, Not detectable; +, 0.05–0.1 ng; ++, 5–10 ng; +++, >10 ng (induced level).

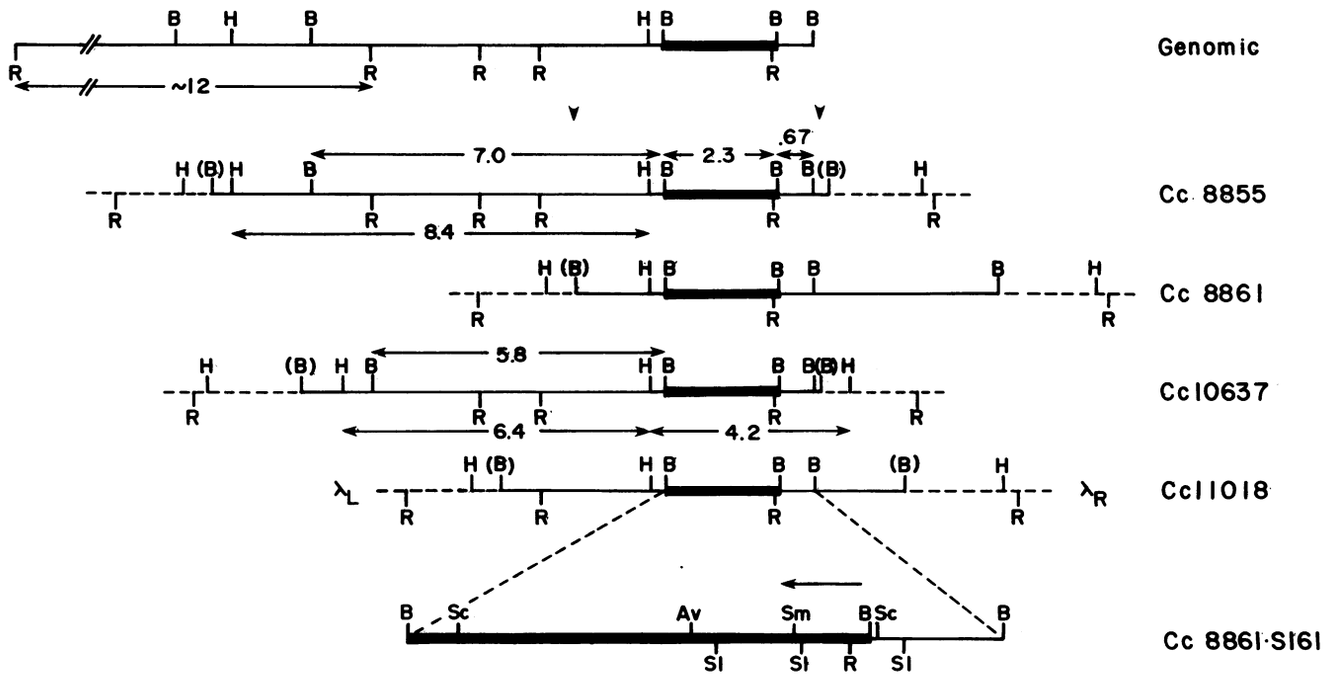

FIG. 3. Restriction maps of 2.3-kb hook protein sequence in λL47.1 hybrid clones, pBR322 subclone, and genome of *C. crescentus*. The CB15 DNA inserts are shown as unbroken lines. The 2.3-kb *Bam*HI fragment containing the hook protein coding sequence is shown as a heavy unbroken line. Arms of the λL47.1 vector are broken lines. CB15 inserts in λL47.1Cc8855 and λL47.1Cc8861 are in the same orientation shown for λL47.1Cc11018; the λL47.1Cc10637 insert is in the opposite orientation. The common 5-kb *Sau*3A–*Sau*3A fragment (see text) is marked by the vertical arrowheads. The proposed direction of hook protein gene transcription on the pBR322·S161 subclone is indicated by the arrow. (B), *Sau*3A where *Bam*HI site is not regenerated in cloning; B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; Sc, *Sac* II; Av, *Ava* I; Sl, *Sal* I; Sm, *Sma* I.

centus, we compared the two proteins by using the mapping technique of Cleveland *et al.* (21). Proteins to be digested were isolated by immunoprecipitation and separated by electrophoresis on 10% $NaDodSO_4$/polyacrylamide gels, and slices from the 72-kDal position on gels were analyzed. Fig. 4 shows the detection of undigested hook protein from a purified sample, the induced *E. coli* S227 subclone, and the polyhook mutant of *C. crescentus* PCM103 by using ^{125}I-labeled anti-hook protein antibody as a probe. The map of antigenic polypeptides generated by partial chymotrypsin digestion of the 72-kDal protein from the S227 subclone was identical to that produced by an immunoprecipitate of hook protein from a purified preparation and from an extract of strain PCM103. The peptide map generated by Pronase was less distinct, but the results support the identification of the cloned gene product as the *C. crescentus* hook protein.

DISCUSSION

The results presented above suggest that the structural information for the hook protein of *C. crescentus* strain CB15 is contained on a 2.3-kb *Bam*HI fragment of the genomic DNA. This conclusion is based primarily on the immunological characterization of the antigen produced by the cloned sequence *in vivo*: (*i*) the four positive λL47.1 clones produced an antigen that reacted in the sandwich screen with anti-hook protein antibody but not with antiflagellin antibody (Fig. 1); (*ii*) pulse-labeled *E. coli* cells infected with two of these clones, λL47.1Cc11018 and λL47.1Cc8861, synthesized a 73-kDal protein which is competitively inhibited by purified hook protein in the radioimmunoassay (Fig. 2; data not shown); (*iii*) only pBR322 and pIN-II subclones that contained the 2.3-kb *Bam*HI fragment synthesized the hook protein (Table 1); and (*iv*) partial proteolysis of the 72-kDal product of the cloned 2.3-kb fragment generated a set of antigenic polypeptides identical to the one produced by purified hook protein (Fig. 4).

Immunologic procedures for identification and characteriza-

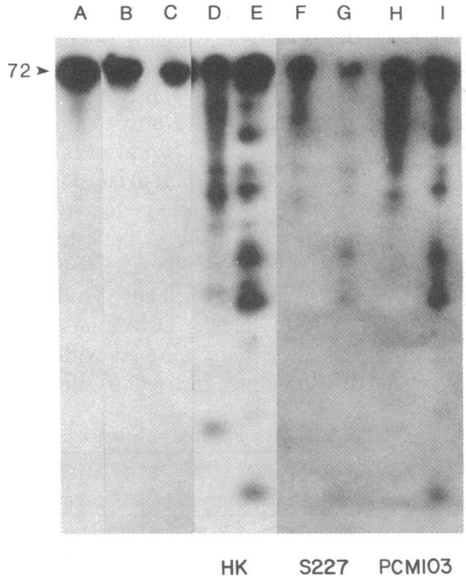

FIG. 4. Peptide maps of cloned hook protein gene product. Purified hook protein (lanes A, D, and E) and hook protein extracts of induced *E. coli* C600/pIN-IICc8861·S227 (lanes B, F, and G) and *C. crescentus* strain PCM103 (lanes C, H, and I) were isolated by immunoprecipitation and electrophoresis on $NaDodSO_4$/polyacrylamide gels. The corresponding gel slices at 72-kDal were incubated in slots of a second 13% $NaDodSO_4$/polyacrylamide gel with either Pronase (0.005 μg in 10 μl; lanes D, F, and H) or chymotrypsin (0.25 μg in 10 μl; lanes E, G, and I). Samples in lanes A–C served as undigested controls. Polypeptides were visualized by autoradiography as described in the text.

tion of genes are useful in systems in which homologous RNA or DNA sequences are not available as probes and complementation tests cannot be readily performed. Success with a radioimmunologic screen like the one used here does depend on the transcription and translation of the cloned DNA to produce a detectable antigenic polypeptide, however. Our experience with the λ bacteriophage vector indicates that individual plaques cannot be screened directly in "shotgun" experiments when a gene is expressed at low levels, even with a very sensitive assay (Fig. 1*A*). The variability of the background made it necessary to screen amplified hybrid phage printed from microtiter wells, and this additional step limits the application of the procedure in screening large genome banks.

Based on the amino acid composition of the hook protein (11), the structural gene should contain approximately 2,076 base pairs, and the results presented above show that most, if not all, of the coding sequence is contained within the 2.3-kb *Bam*HI fragment. Expression of the hook protein gene in *E. coli* at detectable levels depends on the Tc promoter in pBR322 and on the *lpp–lac* promoters in pIN-II (Table 1). This result suggests that either the promoter region is not intact on the cloned fragment or that the promoter or start sequences are not recognized in these cells. Although we cannot distinguish between the two explanations, the failure to detect comparable levels of hook protein when the 2.3-kb sequence was subcloned as part of larger *Bam*HI or *Hin*dIII fragments (Table 1) argues that start sequences for transcription or translation of this gene are not recognized efficiently in the *E. coli* strains tested. Thus, in Cc8861·S161, in which the 0.67-kb *Bam*HI fragment is present between the 5′ end of the gene and the Tc promoter, expression of the 2.3-kb *Bam*HI fragment is reduced drastically (Fig. 1*B*), perhaps as a result of attenuation or termination within the 0.67-kb fragment. Comparisons between levels of hook protein synthesis in phage and plasmid vectors is difficult, but expression of hook protein in λ hybrids, in which the 2.3-kb fragment is part of a larger CB15 DNA insert, is also low and similar to that in Cc8861·S161 (cf. Fig. 1*B* and Table 1).

Although four hook protein clones were identified in a screen of approximately 10 genome equivalents of CB15 DNA, no flagellin A or flagellin B clones were detected. Because the sensitivity of the two immunologic screens is similar, failure to detect flagellin genes could result from the exclusion of these DNA sequences from the clone bank, the inability of the sequences to be transcribed or translated in *E. coli*, or the rapid turnover of the proteins. The apparent requirement that plasmid inserts be in the correct orientation for detectable synthesis of hook protein suggests that the flagellin genes could be present in the λL47.1 clone bank but not expressed at a level sufficient for detection.

Periodic synthesis of the hook protein, as well as induction of flagellin A and flagellin B, requires DNA synthesis (4, 6) and, although other levels of control are also involved in the regulation of these genes (6), we have speculated that the replicating chromosome may act as a "clock" to time initiation of their expression (27). One mechanism for this control would be a cell cycle-dependent rearrangement of flagellar genes, as suggested by the site-specific inversion that occurs during phase variation in *Salmonella* (28). However, preliminary analysis of genomic DNA and cloned sequences with a limited number of restriction enzymes indicates a single arrangement of the hook protein gene in the chromosome (Fig. 3). The possibility remains, however, that any flagellar gene rearrangement leading to a change in the pattern of expression would be transitory and, as such, difficult to detect in Southern blots of total genomic DNA from exponential *C. crescentus* cells.

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