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Rate, origin, and bidirectionality of *Caulobacter* chromosome replication as determined by pulsed-field gel electrophoresis

(development/DNA replication)

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ABSTRACT **Cell division in *Caulobacter crescentus* yields progeny cells that differ with respect to cell structure and developmental program. Chromosome replication initiates in the daughter stalked cell but is repressed in the daughter swarmer cell until later in the cell cycle. To study cell-type-specific DNA initiation, chromosome replication was directly analyzed by pulsed-field gel electrophoresis. Analysis of *Dra* I restriction fragments of DNA taken at various times from synchronized cell cultures labeled with 2′-deoxy[³H]-guanosine has allowed us to determine the origin of DNA replication, the rate and direction of fork movement, and the order of gene replication. The first labeled *Dra* I fragment to appear contains the site of replication initiation. Based on the correlation of the physical and genetic maps derived by Ely and Gerardot [Ely, B. & Gerardot, C. J. (1988) *Gene* 68, 323–333], the origin was localized to a 305-kilobase fragment containing the *rrnA* gene. Furthermore, the sequential replication through unmapped *Dra* I fragments has enabled us to localize their positions on the genome. The order of appearance of labeled restriction fragments revealed that the chromosome replicates bidirectionally at a fork movement rate of 21 kilobases per minute.**

Cell division in *Caulobacter crescentus* yields progeny cells that differ with respect to cell structure, developmental program, and the time of initiation of DNA replication (1, 2). Each division yields a motile swarmer cell and a sessile stalked cell (Fig. 1). Chromosome replication initiates in the daughter stalked cell but is repressed in the daughter swarmer cell until its transition into a stalked cell later in the cell cycle (3, 4). *C. crescentus* is ideal for a study of the temporal regulation of chromosome replication because it has a well-characterized cell cycle, only undergoes one round of replication per cycle, and is amenable to genetic and molecular analysis of cell-type-specific events.

Chromosome replication in bacteria is tightly regulated, and cell cycle control of gene expression involving replication-associated proteins indicates that timing is critical (5, 6). The trigger that starts the process may involve phospholipid synthesis (7, 8), accumulation of growth-dependent positively acting initiators (9), and selective transcription of genes involved in replication initiation at the origin (10, 11). Cell growth and proliferation depend in large measure on events leading to duplication of the chromosome and cell division (12, 13). Conservation of the minimal chromosomal origin of replication in several bacterial species (12–14) suggests that common mechanisms may be involved in regulation of this process.

To begin a study of the temporal control of initiation of replication and to identify the cellular factors involved in this regulation, we have used pulsed-field gel electrophoresis (PFGE; refs. 15–18) of the *C. crescentus* chromosome to directly determine the origin of DNA replication and the rate of replication and to demonstrate bidirectional replication. A physical map of *Dra* I restriction fragments has recently been correlated to the *C. crescentus* genetic map (18). In the experiments described here, synchronized populations of swarmer cells were labeled *in vivo* with 2′-deoxy[³H]-guanosine and allowed to proceed through the cell cycle. The process of DNA replication was followed at timed intervals by observing labeled chromosomal *Dra* I restriction fragments in pulsed-field gels. The first labeled *Dra* I fragments to appear represent the initiation of replication. The order and time of appearance of labeled restriction fragments relative to the origin reveal the direction and rate of chromosomal replication. Regression analysis of these data has allowed us to locate the origin of DNA replication and to demonstrate bidirectional replication with a fork movement rate of 21 kilobases (kb) per minute.

MATERIALS AND METHODS

Synchronization of Cell Cultures. *C. crescentus* CB15N was grown overnight in PYE broth (19), transferred to modified minimal M2 broth (20), and grown overnight at 30°C. The culture was then diluted ≈100-fold and grown in minimal broth at 30°C to an OD_{660} = 0.9, and swarmer cells were isolated by density gradient centrifugation through Ludox (DuPont) as described by Evinger and Agabian (21). Purified (>90%) swarmer cells were diluted to an OD_{660} = 0.2 in minimal broth and incubated in the presence of 2′-deoxy-[8-³H]guanosine (ICN) at 5 μCi/ml (1 μCi = 37 kBq).

Analysis of Labeled DNA. The incorporation of deoxy[³H]-guanosine into DNA was measured by removing aliquots (100 μl) of cells at 10-min intervals, hydrolyzing cellular RNA in 0.5 M NaOH at 60°C, and then neutralizing the extract with 0.5 M HCl. Trichloroacetic acid was then added [final concentration, 20% (wt/vol)] to the extracts and the radioactivity of the precipitates was determined with a Packard Tri-Carb liquid scintillation counter. Analysis of these data (not shown) revealed a brief lag, followed by a linear incorporation of label throughout the S phase of the cell cycle.

To analyze the chromosomal distribution of label, aliquots (10 ml) of cells were taken at 10-min intervals, diluted with an equal volume of cold Pett IV buffer (10 mM Tris, pH 7.6/1 M NaCl) and collected by centrifugation at 10,000 × *g* for 10 min. Cells were washed twice with cold Pett IV buffer and resuspended in the same buffer in a final volume of 0.5 ml. The cell suspensions were briefly warmed to 37°C and mixed with equal volumes of 1% (wt/vol) agarose type IV (Sigma) and cast into molds (15). The agarose plugs were prepared for electrophoresis as described by Ely and Gerardot (18). The DNA embedded in the plugs was digested for 7 hr at 37°C with 33 units of the restriction enzyme *Dra* I (Boehringer Mann-

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Abbreviation: PFGE, pulsed-field gel electrophoresis.
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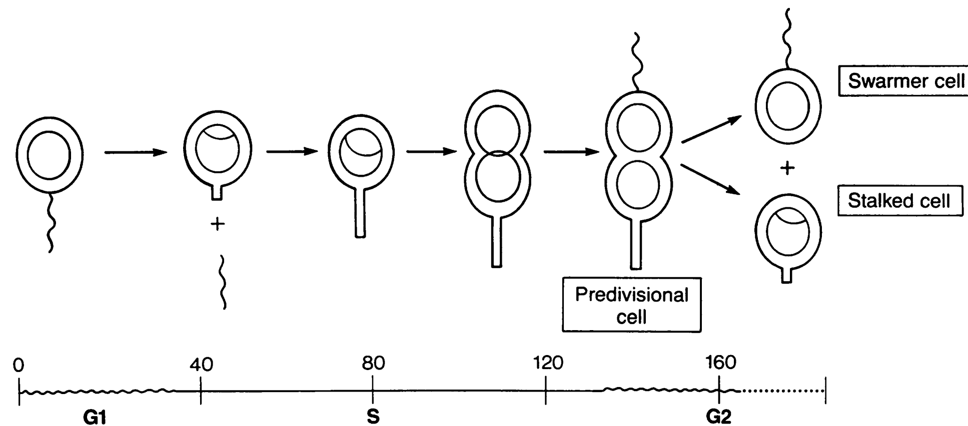

FIG. 1. *C. crescentus* cell cycle. Swarmer cells in G_1 phase are unable to replicate their chromosome. The transition to the stalked cell is concomitant with ejection of the single polar flagellum, initiation of chromosome replication, and formation of the stalk (S phase). Replication proceeds bidirectionally until resolution and segregation of chromosomes into the daughter cells (G_2 phase). One cell cycle completes upon division at ≈160 min in minimal medium.

heim). The agarose plugs were then placed into wells of a 1% agarose gel. PFGE was carried out as described (18). The gels were stained with ethidium bromide and photographed with Kodak type 55 film. The gels were then treated with EN^3HANCE (NEN) for 2 hr, soaked in water for 2 hr, and dried under vacuum at 80°C on a gel dryer for 1 hr. Dried gels were exposed directly to Kodak XAR autoradiography film for 2 days at −70°C with intensifying screens.

RESULTS

PFGE of *C. crescentus* DNA from Various Times of the Cell Cycle. The *C. crescentus* cell cycle is shown schematically in Fig. 1. The progeny swarmer cell is unable to initiate DNA replication until the swarmer → stalked cell transition. The initiation of chromosome replication in this cell occurs just prior to, or concurrently with, ejection of the flagellum and the subsequent formation of the stalk (3). Under the growth conditions used in the experiments described here, the generation time was 160 min and the swarmer → stalked cell transition occurred at ≈40 min into the cell cycle (Fig. 1).

To determine the precise time and start site of replication initiation, synchronous cultures were grown in the presence of deoxy[^{3}H]guanosine and aliquots were removed at 10-min intervals ranging from 0 min (swarmer cells) to 100 min (dividing cells). The labeled cells from each time point were embedded in 1% agarose, lysed *in situ*, and digested with the restriction endonuclease *Dra* I (TTT ↓ AAA). This enzyme cuts *C. crescentus* DNA infrequently due to the organism's high G+C content (18), and digestion results in 35 bands ranging from 10 to 400 kb (Fig. 2). The digested chromosome fragments were then subjected to pulsed-field electrophoresis through a 1% agarose matrix for 19 hr with various pulse times (see below and Fig. 2 legend). The lanes from left to right in each gel represent successive time points in the cell cycle; the phage λ DNA ladder partially visible in the left-most lane serves as size reference (50, 100, and 150 kb). The number of cells present at the earliest time point was the same as that at the latest time point. Bands that show brighter fluorescence relative to other bands indicate that more than one fragment of a given size is present. This is true of bands at 17, 105, 130, and 145 kb.

The pulse time determines the distance of migration of each fragment as well as the resolution of similarly sized fragments. Longer pulse times result in greater separation of the larger fragments. Resolution of the largest fragments (145–400 kb) was obtained by using a pulse time of 20 sec (Fig. 2*A*). A 10-sec pulse time gave optimal resolution in the range 50–230 kb, with the larger fragments condensed at the top of the gel (Fig. 2*B*). Resolution of fragments ranging from 23 to 190 kb was achieved with a 7-sec pulse time (Fig. 2*C*).

Determination of Replication Origin and Direction. The ethidium bromide-stained gels shown in Fig. 2 were subjected to autoradiography in order to visualize the restriction fragments that had incorporated deoxy[^{3}H]guanosine at each time point in the cell cycle (Fig. 3). The film exposure is a direct reproduction of the band configuration seen with staining, allowing easy comparison of bands both labeled and stained. No labeled fragments were present in lanes containing DNA from cells 10 or 20 min into the cell cycle (Fig. 3). The first labeled fragments, of 305 and 105 kb, appeared at 30 min (Fig. 3 *A*, *C*, and *D*). This indicates the time and location of replication initiation of the *C. crescentus* chromosome. Continuous labeling allows for detection of the first appearance of a labeled restriction fragment as the essential criterion to determine rate of replication as well as serving as an internal control for quality of the synchrony, and also allows comparison of several different experiments. Pulse-labeled cultures were also tested and showed the initial appearance of the 305- and 105-kb *Dra* I fragments 30 min into the cell cycle (data not shown). As originally constructed by Ely and Gerardot (18), the physical map of *Dra* I fragments in *C. crescentus* places these two fragments at least 550 kb apart. However, there are two 105-kb *Dra* I fragments in *C. crescentus*, one of which has not been ordered into the physical map. The experiments described here demonstrate that the second 105-kb fragment is next to the 305-kb fragment (see Fig. 5). No known genes have been mapped to this 105-kb fragment, so we cannot tell whether it is to the left or the right of the 305-kb fragment.

The order of appearance of labeled restriction fragments shows that after initiation of chromosomal replication within the 305-kb fragment, the replication fork moves to the 115-kb fragment next and then to the 155-kb fragment. The next bands to appear are the 75-kb fragment and the 35-kb fragment, followed by the 190-kb and 145-kb fragments, and soon after by the 230-kb fragment. Bidirectional fork movement is indicated because the order of appearance of the labeled restriction fragments occurs both clockwise and counterclockwise from the apparent origin within the 305-kb fragment (Fig. 3 *A*–*C*). Replication is nearly completed by 100 min (late S phase) (Fig. 3*D*). The *Dra* I fragments that appear last are 130 kb (Fig. 3*B*) and 320 kb (data not shown), both of which are ≈1500 kb from the origin in opposite directions. The data suggest that a single replication cycle is completed ≈80 min after initiation.

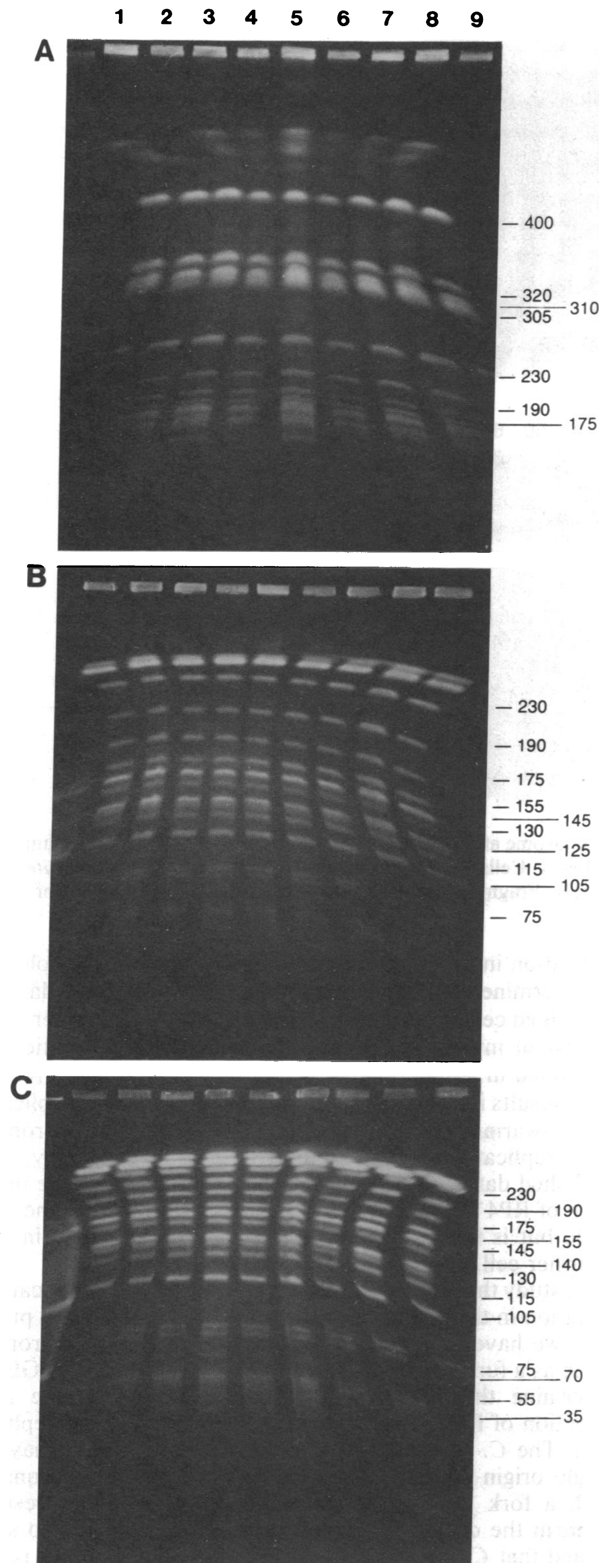

FIG. 2. Ethidium bromide-stained pulsed-field agarose gels. *C. crescentus* cultures were synchronized and an aliquot was taken every 10 min from 20 to 100 min (lanes 1–9, respectively) of the swarmer cell cycle (see Fig. 1). The *Dra* I-treated DNA samples were subjected to PFGE, with pulse times as indicated below, for 19 hr at 9°C, and 230 V in 0.5× TBE buffer (1× TBE is 89 mM Tris/89 mM boric acid/1 mM EDTA). After electrophoresis, gels were stained with ethidium bromide (1 μg/ml) and photographed using a long-wave UV transilluminator. (*A*) Pulse time, 20 sec; *Dra* I restriction fragments ranging from 400 to 175 kb are resolved as indicated. (*B*) Pulse time, 10 sec; resolution, 230 to 75 kb. (*C*) Pulse time, 7.5 sec; resolution, 230 to 35 kb.

Bidirectional Fork Movement and Rate Determination. The location of the origin of replication, the time of initiation, and the rate of DNA synthesis were estimated by regression analysis (Fig. 4). The physical distance from the putative origin within the 305-kb band to each *Dra* I fragment was plotted as a function of the time of replication of that fragment. Fragments on the counterclockwise fork were arbitrarily denoted negative (−), and those on the clockwise fork were denoted positive (+). The time of first appearance of each band was plotted on the *x*-axis. The appearance of a band indicates that the replication fork has passed completely through the fragment, that is, beyond the end of the fragment that is farthest from the origin. The distance from the origin-distal end of the fragment to the putative origin was plotted on the *y*-axis. The distances are derived from the physical map of Ely and Gerardot (18), and the times of appearance of labeled restriction fragments were measured in our experiments. The correlation coefficients for the two forks were 0.99 (+) and 0.96 (−), indicating agreement between the order of fragments in the physical map and time-course data.

A least-squares regression line was drawn through the points from each replicating fork (Fig. 4). The slopes gave a fork movement rate of 21.5 kb/min (+) and 20.5 kb/min (−), yielding a total replication rate of 42 kb/min in a minimal medium.

The point of intersection gives the timing and location of the start of replication. In Fig. 4, the intersection was arbitrarily set equal to zero. The analysis was done twice, with the 105-kb fragment on either the positive or the negative side of the 305-kb fragment. With the 105-kb fragment on the positive side, the origin is located ≈130 kb from the 305 kb/105 kb border. Placement of the 105-kb fragment on the negative side results in an origin located ≈60 kb away from the 305 kb/105 kb border (data not shown). The location of the 105-kb fragment has no effect on the rate of replication or the timing of initiation. Extrapolation of the regression lines to the origin indicates that the initiation of replication occurs at approximately the same time as the ejection of the flagellum and the initiation of stalk biosynthesis (Fig. 1).

Chromosome Replication Map. Genetic analysis together with PFGE separation of *Dra* I restriction fragments enabled Ely and Gerardot (18) to construct a physical map of the *C. crescentus* chromosome (Fig. 5). Genetic loci were assigned to specific large *Dra* I restriction fragments by PFGE analysis of DNA from a large number of mutants containing Tn*5* insertions at known map positions. By measuring the time of appearance of newly replicated portions of the chromosome, we have been able to determine the initiation site and direction of replication fork movement with respect to the physical and genetic map (Fig. 5). The origin was placed within the 305-kb fragment near the border with the 105-kb fragment on the positive side. This location of the origin is consistent with an earlier report (4) showing that replication begins near the ribosomal RNA gene (*rrnA*) within the 305-kb fragment. The origin is also located near the *rrnB* gene, which has been placed in the 35-kb *Dra* I fragment (ref. 18; A.D. and K. Amemiya, unpublished data), consistent with a placement of the 35-kb fragment near the 305-kb fragment on the positive side. Other possible locations for the 105-kb and 35-kb fragments are indicated on the negative side of the 305-kb fragment.

Previous work has not mapped the location of the 75-kb *Dra* I restriction fragment. However, our experiments allowed placement of the 75-kb fragment on the negative side of the origin between the 155-kb and 145-kb fragments. The 75-kb fragment is replicated after the 155-kb fragment containing *hunG* and before the 145-kb fragment containing *serA* (Figs. 3 and 5). This is consistent with genetic mapping data placing the *purB* gene between *hunG* and *serA* and within the 75-kb fragment.

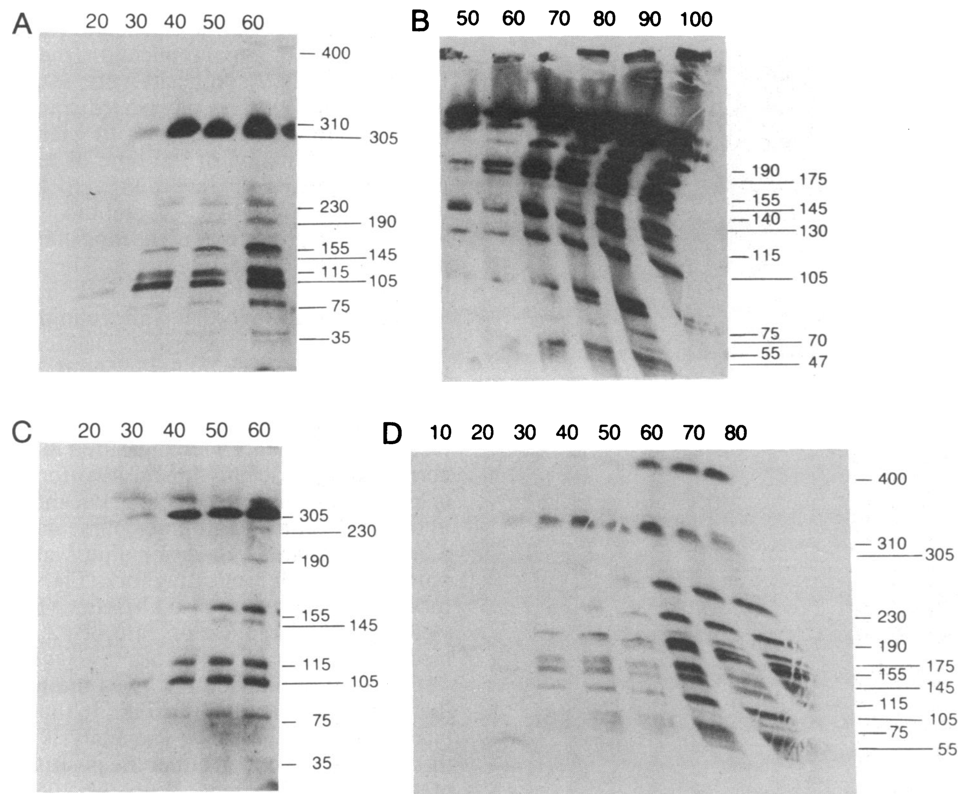

FIG. 3. Autoradiographs of deoxy[^{3}H]guanosine-labeled *C. crescentus* chromosome after separation of *Dra* I fragments by PFGE. Numbers above the lanes indicate time in minutes in the swarmer cell cycle as shown in Fig. 1. Cells were labeled continuously as described in *Materials and Methods*. Pulse time was 20 sec (*A*), 10 sec (*B*), 7.5 sec (*C*), or 20 sec (*D*). Fragment sizes in kilobases are indicated at right of each autoradiograph.

DISCUSSION

C. crescentus is unique in that upon cell division, the daughter stalked cell immediately reinitiates chromosome replication, whereas the daughter swarmer cell is unable to initiate replication until later in the cell cycle. This natural asymmetry allows for observation of the events relevant to replication initiation without modifying cellular physiology. To determine why replication initiation occurs in the daughter stalked cell but is repressed in the daughter swarmer cell, one might investigate whether plasmid DNA replication is controlled in parallel with chromosome replication. Preliminary results indicate that the plasmid RP4 is able to replicate in the swarmer cell at a time when the initiation of chromosome replication is repressed (A.D. and B. S. Loewy, unpublished data), suggesting that the machinery for the initiation of RP4 DNA replication is present throughout the cell cycle but is inaccessible to the chromosomal origin in the swarmer cell.

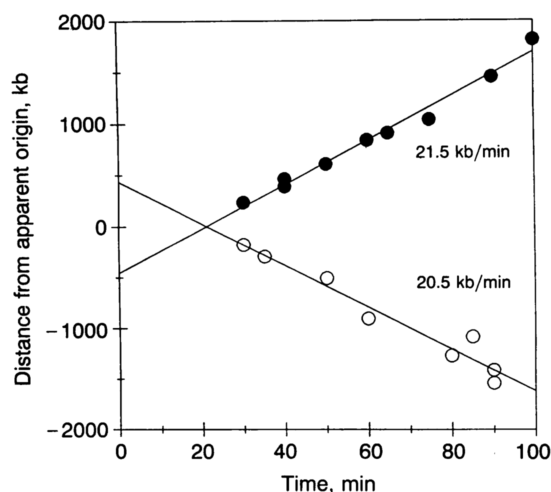

FIG. 4. The apparent origin, time of initiation, and rate of DNA synthesis estimated by regression analysis. The time of first appearance of each fragment was plotted as a function of distance from the origin in either a clockwise (−) direction or counterclockwise (+) direction. The replication rate in each direction from the origin was calculated from the slopes. The intersection of the two regression lines represents the time of initiation of chromosome replication within the 305-kb *Dra* I fragment.

To study the mechanism and regulation of DNA replication initiation in the *Caulobacter* swarmer and stalked cell progeny, we have begun an analysis of the replicating chromosome as a function of the cell cycle. We have used PFGE to determine the origin of DNA replication, the rate and direction of fork movement, and the order of gene replication. The *C. crescentus* chromosome was found to have a single origin of replication and to replicate bidirectionally with a fork movement rate of 21 kb/min. These results confirm the conclusions of Lott *et al.* (4), which also suggested that *C. crescentus* DNA has a single origin of replication near the ribosomal gene cluster and that replication appears to proceed bidirectionally. The localization of the origin of replication to the vicinity of a ribosomal RNA operon in *C. crescentus* allows use of rRNA clones (22, 25) as probes to isolate the origin from a cosmid library.

Earlier attempts to correlate *Dra* I restriction fragments with positions on the *C. crescentus* genetic and physical map have been hindered by the lack of mutations in certain regions of the chromosome (18). The ability to determine the time of replication of a given restriction fragment will now aid in positioning it within the physical map by the use of regression

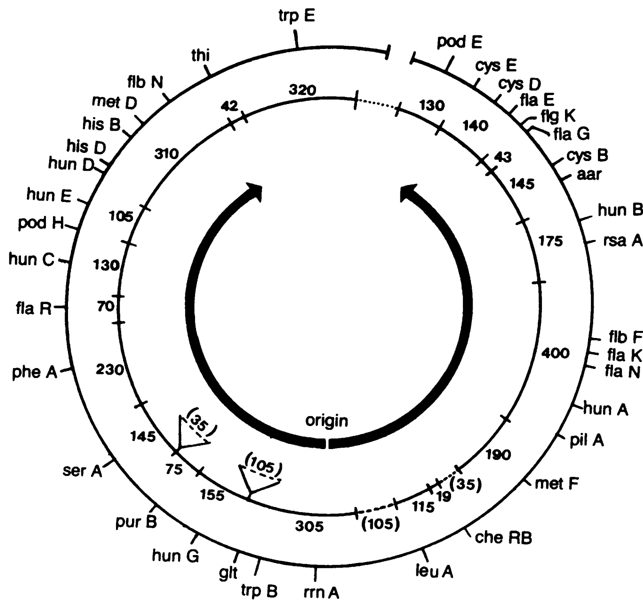

FIG. 5. Initiation site and direction of replication of the *C. crescentus* chromosome. The sizes and relative positions of *Dra* I restriction fragments in relation to the genetic map were determined by Ely and Gerardot (18), except as indicated in the text. The location of the origin of replication within the 305-kb fragment was determined from the regression analysis shown in Fig. 4. The presumptive locations of the 105- and 35-kb fragments are shown by dotted lines, within the restriction map (inner circle); alternative positions are indicated inside the restriction map. The direction and extent of experimentally determined replication fork movement are shown by the heavy line.

analysis as shown in Fig. 4. This method was used to locate the position of the 75-kb fragment. It was also used to locate the 105-kb and the 35-kb fragments, although we cannot ascertain whether they are in the clockwise or counterclockwise position with respect to the origin (Fig. 5).

It has not yet been possible to determine whether or not the *C. crescentus* chromosome is circular, because a genetically silent region exists between genes on the 320-kb and 130-kb *Dra* I fragments (Fig. 5; refs. 23 and 24). It should be possible to determine whether the chromosome is circular by using PFGE of fragments generated by several different restriction enzymes to establish whether or not replication proceeds through this genetically silent region.

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