

# Roles of novobiocin-sensitive topoisomerases in chloroplast DNA replication in *Chlamydomonas reinhardtii*

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## ABSTRACT

**We have examined DNA replication in *Chlamydomonas reinhardtii* chloroplasts *in vivo* when chloroplast type II topoisomerases are inactivated with sublethal doses of novobiocin. DNA replication is at first inhibited under these conditions. However, after a delay of several hours, chloroplast chromosomes initiate a novobiocin-insensitive mode of DNA replication. This replication starts preferentially near a hotspot of recombination in the large inverted repeats, instead of from the normal chloroplast origins, *oriA* and *oriB*. It replicates one, but not the other single-copy region of the chloroplast chromosome. We speculate that novobiocin-insensitive DNA replication in chloroplasts requires recombination in this preferred initiation region.**

## INTRODUCTION

The single chloroplast of the unicellular green alga *Chlamydomonas reinhardtii* contains 50–100 copies of a circular chromosome. Each chromosome is composed of two genetically distinct, single-copy regions (SCRs) which encode many proteins involved in photosynthesis and chloroplast protein synthesis. The SCRs are separated by two identical copies of a large inverted repeat (IR) sequence which contains the rRNA cistrons and *psbA*, a gene encoding one component of the photosystem II complex (Fig. 1A) (reviewed in ref. 1).

Chloroplast DNA is maintained at a relatively stable average copy number under normal growth conditions. Two *de novo* origins of DNA replication, *oriA* and *oriB*, are located close to one another in one SCR (Fig. 1A) (2). Replication from these origins starts with the formation of displacement loops (D-loops), followed by bidirectional, double-strand DNA synthesis. Negatively supercoiled plasmids containing *oriA* can replicate *in vitro* in algal extracts (3). Several partially purified proteins from such extracts bind specifically to the *oriA* region (4, 5, 6). In many respects, these *de novo* chloroplast origins resemble origins of *E. coli* and its plasmids and phages (3, 7). Furthermore, some chloroplast and nuclear-encoded proteins of *C. reinhardtii* resemble proteins involved in *E. coli* DNA replication (8).

Chloroplasts contain an ATP-dependent, type II topoisomerase with a similar activity as that of bacterial DNA gyrase (9,10). In bacteria, DNA gyrase (together with other factors) maintains superhelical tension of chromosomal DNA (11, 12). This enzyme

is important for initiation of DNA replication and movement of replication forks (for reviews, see 13, 14, 15). A second type II enzyme called topoisomerase IV is required to separate intertwined, covalently closed daughter DNA molecules (16, 17) prior to segregation (18, 19, 20). Both of these type II topoisomerases are inhibited by novobiocin (21), a competitive inhibitor of ATP-binding (22, 23).

Previous results have shown that chloroplast DNA is under superhelical tension *in vivo*. Nicking of chloroplast DNA by  $\gamma$ -ray irradiation or inhibition of type II chloroplast topoisomerases with novobiocin both reduce superhelical tension as measured by the intercalation of the psoralen derivative HMT (4'-hydroxymethyl-4,5',8-trimethylpsoralen) into chloroplast DNA (24, 25, 26). The latter result suggests that novobiocin-sensitive topoisomerases play similar roles in modulating superhelical tension of DNA in bacteria and in chloroplasts. Since we do not know whether novobiocin inhibits more than one type II chloroplast topoisomerase, we refer to these enzymes in the plural.

Here we describe chloroplast DNA replication *in vivo*, when chloroplast type II topoisomerases are inhibited with novobiocin. We show that DNA synthesis is at first inhibited under these conditions. At later times, however, chloroplast chromosomes initiate a novobiocin-insensitive mode of DNA synthesis preferentially from a region that is different from *oriA* and *oriB*. This initiation region is located in the IRs close to a hotspot of recombination (27). In the presence of novobiocin, this replication mode results in only partial replication of the chloroplast chromosome.

## MATERIALS AND METHODS

### Algal strains and growth conditions

*C. reinhardtii* strains *cc125* (wt<sup>+</sup>), *cc278 cw-15* (cell wall<sup>-</sup>), FUD7, and *ac-u-c-2-43* were obtained from the *Chlamydomonas* Genetics Center, Duke University. Medium and culture conditions have been described previously (28). The strain *cc278 cw-15* was used to prepare total DNA for Southern blot analyses.

### Preparation of total algal DNA and Southern transfer

Preparation of total DNA from *C. reinhardtii* has been described previously (25). Southern blots were done and <sup>32</sup>P-labeled DNA probes were prepared by replacement synthesis as described (29).

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### Mapping of chloroplast *Hpa* II restriction fragments

Southern blots of purified chloroplast DNA (30), digested with *Hpa* II, were probed with cloned chloroplast restriction fragments from different regions of the chromosome (shown in Fig. 1A). In addition, isolated chloroplast *Hpa* II fragments were used to probe Southern blots of digests by other restriction enzymes of purified chloroplast DNA. From the combined results of these experiments, the largest chloroplast *Hpa* II restriction fragments were mapped relative to the *Eco* RI and *Bam* HI restriction maps of the chromosome (Fig. 1A) (31). These *Hpa* II fragments are distributed over all regions of the chromosome and together, comprise more than 50% of the chloroplast genome.

### *In vivo* labeling of chloroplast DNA

Exponentially-growing cells ( $A_{660nm}=0.1-0.2$ ) were treated with novobiocin (final concentration 450  $\mu\text{g/ml}$ ) for varying times prior to labeling. Cells (5–10 total  $A_{660}$  units) were harvested by centrifugation ( $3000\times g$ , 3 min), washed in 5 ml of phosphate-free medium (Bold's Basic Medium, without bacto-peptone, yeast extract, and phosphate supplements), and repelleted. The cells were resuspended in phosphate-free medium and incubated 5 min at room temperature prior to labeling with  $^{32}\text{P}$ . To each aliquot of cells, 0.5 mCi of  $\text{H}_3^{32}\text{PO}_4$  were added and the cells were incubated 30 min at room temperature. The  $^{32}\text{P}$ -labeled cells were added to 30–50 total  $A_{660}$  units of unlabeled 'carrier cells' grown in Bold's Basic Medium. 'Cold'  $\text{NaH}_2\text{PO}_4$  was added (final concentration 100 mM) and the cells were kept on ice for 2 min. Total DNA was then prepared as described above.

The DNA concentration of samples was determined by UV absorbance and was further quantitated after agarose gel electrophoresis. Approximately 1  $\mu\text{g}$  of each DNA sample was compared to standards of known DNA concentration after staining the gel with ethidium.

Incorporation of  $^{32}\text{P}$  into preparations of total DNA was determined by TCA precipitation (29) of  $\sim 1 \mu\text{g}$  of each sample. Since the specific activities (i.e. cpm/ $\mu\text{g}$  of total DNA) of most DNA samples +/- novobiocin were similar ( $\sim 5\times 10^4-2\times 10^5$  cpm/ $\mu\text{g}$  DNA), equal amounts (20–30  $\mu\text{g}$ ) of total DNA were digested with *Hpa* II ( $\sim 20$  u) for 8–16 hr at 37°C. Restriction fragments were separated by electrophoresis (30 cm horizontal slab gels, 0.8% agarose in 40 mM Tris-acetate pH8.2, 2 mM EDTA) for 14–16 hr at 60 V. Agarose gels were fixed in 7% TCA for 30 min, pressed to complete dryness under paper towels, and autoradiographed for 4–48 hr at room temperature.

### Enzymes and chemicals

All restriction enzymes as well as Klenow fragment (large fragment of *E. coli* DNA polymerase I) and T4 DNA polymerase were from Promega. Novobiocin and kanamycin were from Sigma;  $\text{H}_3^{32}\text{PO}_4$  (285 Ci/mg) was from ICN, [ $\alpha$ - $^{32}\text{P}$ ] dCTP (800 Ci/mmol) from NEN.

## RESULTS

### An *in vivo* assay for chloroplast DNA replication

In order to examine chloroplast DNA replication *in vivo*, it is necessary to distinguish nascent chloroplast DNA from that of the nuclear and mitochondrial chromosomes. We have developed a simple method to identify replicating chloroplast DNA after labeling growing *C. reinhardtii* cells with  $^{32}\text{P}$ . Under the conditions used here, cytoplasmic factors which may influence organelle DNA replication can enter the chloroplast.

The AT-rich chloroplast DNA of *C. reinhardtii* contains relatively fewer recognition sites for the restriction endonuclease *Hpa* II (5' CCGG 3') than nuclear DNA with its higher GC-content. Most large *Hpa* II restriction fragments are derived from chloroplast DNA and can be resolved from smaller, nuclear *Hpa* II fragments by agarose gel electrophoresis (Fig. 1B)(32). The location on the chloroplast chromosome of several of these large *Hpa* II fragments is shown in Figure 1A.

To detect nascent chloroplast DNA, an exponential culture of algal cells was transferred to and allowed to grow for 30 min in a low-phosphate medium containing high specific-activity  $^{32}\text{P}$ . Algal cells incorporate this label into nascent DNA, RNA, and other compounds. Total DNA was purified from  $^{32}\text{P}$ -labeled cells, digested with *Hpa* II, and the restriction fragments were separated by electrophoresis. For comparison, unlabeled, density-gradient-purified chloroplast DNA was also digested with *Hpa* II.

Both nuclear and chloroplast DNA were synthesized during the labeling period *in vivo* (Fig. 1C). All chloroplast *Hpa* II restriction fragments which can be unambiguously identified were labeled with  $^{32}\text{P}$  under these conditions. A densitometric scan of the autoradiograph in Figure 1C shows that incorporation of  $^{32}\text{P}$  was approximately proportional to the size of the chloroplast *Hpa* II fragments (Fig. 1D). This is expected for an asynchronously-dividing population of cells since chloroplast chromosomes are replicated at random throughout the cell cycle (33).

### Inactivation of novobiocin-sensitive topoisomerases rapidly inhibits chloroplast DNA replication *in vivo*

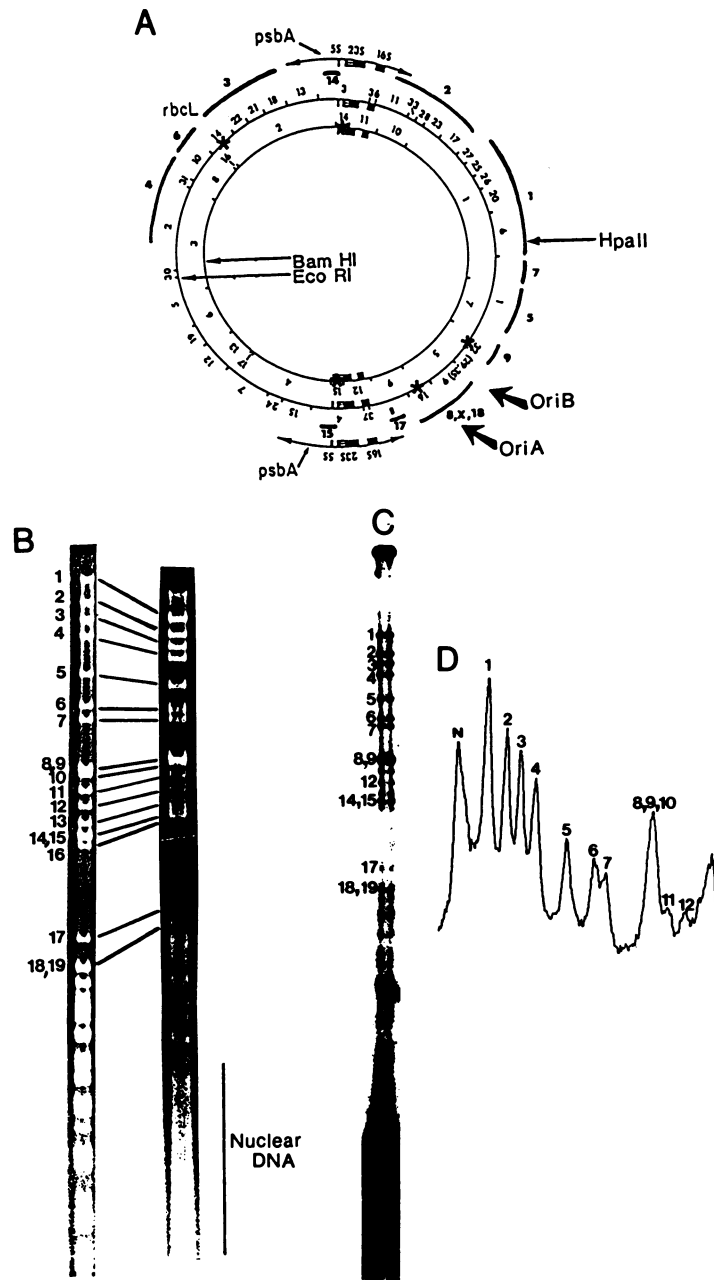
Previously, we have shown that superhelical tension in chloroplast DNA is reduced *in vivo* within 1 hr after the addition of novobiocin to growing cells (25, and our additional unpublished results). Therefore, we tested the effects of novobiocin on chloroplast DNA synthesis within a similar time frame. Novobiocin, at the concentrations employed here has little effect on growth of the algae for at least 24 hr (2–3 cell generations). During the next 24 hr, growth of novobiocin-treated cells slows relative to untreated cells and then stops. At later times (>96 hr) when the novobiocin is presumably inactivated, cells resume growth and reach the same saturated cell density as untreated cultures. Thus, the cells are not killed at the concentrations of the drug used here and we conclude that novobiocin has little if any effect on nuclear and mitochondrial functions during the 24 hr period when chloroplast DNA synthesis measurements were made.

Replicating DNA was labeled with  $^{32}\text{P}$  in exponentially-growing cells after the addition of novobiocin. Total DNA, prepared from drug-treated and from untreated control cells, was digested with *Hpa* II and separated by electrophoresis as described above. Incorporation of  $^{32}\text{P}$  into all distinguishable chloroplast *Hpa* II restriction fragments was dramatically reduced within 1 hr of addition of novobiocin; Figure 2A shows a representative example where chloroplast DNA synthesis was reduced within  $\sim 45$  min of addition of the drug. Similar results were obtained  $\sim 2.5$  hr after addition of novobiocin (Fig. 2B). As expected, novobiocin had little, if any, effect on nuclear DNA synthesis under these conditions (as shown by the small *Hpa* II fragments in Fig. 2). In other experiments (data not shown), we have found that chloroplast DNA synthesis is inhibited to a similar extent by 250  $\mu\text{g/ml}$  novobiocin. At this concentration, type II topoisomerases are the only known target for novobiocin in

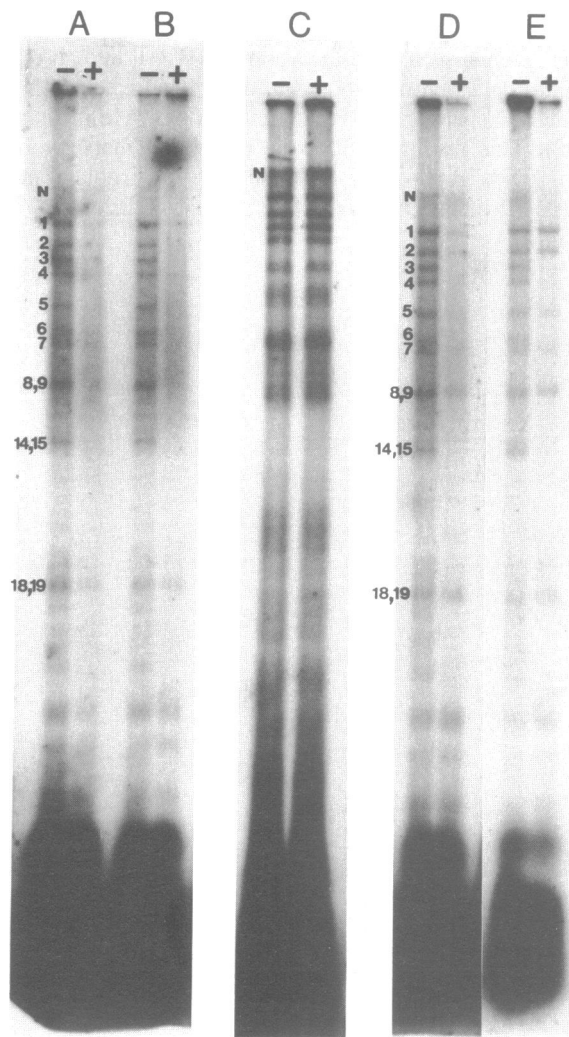
*Chlamydomonas* (10, 25), indicating that novobiocin-sensitive topoisomerases are required for normal chloroplast DNA replication.

To address the possibility that novobiocin might inhibit DNA synthesis indirectly by affecting the production or decay of chloroplast proteins, we tested the effect of kanamycin which specifically inhibits chloroplast protein synthesis. Kanamycin has

no effect on superhelical tension in chloroplast DNA as measured by HMT intercalation (25). Incorporation of  $^{32}\text{P}$  into all regions of the chloroplast chromosome was unaffected when cells were treated with kanamycin (a representative example is shown in Fig. 2C). This result excludes the possibility that novobiocin inhibits DNA replication by inhibiting chloroplast protein synthesis. Proteins required for normal chloroplast DNA



**Figure 1.** *In vivo* labeling of chloroplast DNA. **A.** A circular restriction map for the enzymes *Eco* RI and *Bam* HI (from ref. 1). The inverted repeat regions (IRs) are bracketed by arrowheads. The locations of the two chloroplast origins, *oriA* and *oriB* are indicated by arrows. Chloroplast *Hpa* II restriction fragments (see Materials & Methods) are shown on the outside of the map and are numbered according to size (as in ref. 30). Cloned chloroplast restriction fragments used as probes are indicated with asterisks. **B.** Purified chloroplast DNA was digested with *Hpa* II and separated by agarose gel electrophoresis; the gel was stained with ethidium and photographed. The major chloroplast restriction fragments are numbered according to size and correspond to *Hpa* II fragments shown in A. Total DNA was prepared from *cc125* cells as described and was digested with *Hpa* II. The DNA fragments were resolved by electrophoresis, stained with ethidium and photographed. Nuclear *Hpa* II restriction fragments are indicated. **C.** An autoradiograph of  $^{32}\text{P}$ -labeled total DNA digested with *Hpa* II. Algal cells were labeled with  $^{32}\text{P}$  for 30 min and total DNA was prepared as above. The *Hpa* II-digested,  $^{32}\text{P}$ -labeled DNA was then separated by electrophoresis as in B and autoradiographed (12.5 hr at room temperature). **D.** A densitometric scan of the autoradiograph shown in C. (*Hpa* II fragments 1–12). N indicates high molecular weight nuclear DNA fragments.



**Figure 2.** Novobiocin affects chloroplast DNA synthesis *in vivo*. Total DNA, labeled *in vivo* with  $^{32}\text{P}$ , was prepared from *cc125* cells grown in the presence (+) or absence (-) of 450  $\mu\text{g}/\text{ml}$  novobiocin for A. ~45 min, B. ~2.5 hr, D. ~8.5 hr, and E. ~24.5 hr. C. Total DNA, labeled with  $^{32}\text{P}$  as in A, was prepared from cells grown in the presence (+) or absence (-) of 100  $\mu\text{g}/\text{ml}$  kanamycin for 2 hr. Chloroplast *Hpa* II restriction fragments were separated by electrophoresis and are numbered as in Fig. 1; N indicates nuclear DNA fragments. For panel A., novobiocin was added to the culture and cells were immediately harvested for labeling. Cells were labeled for 30 min in low-phosphate medium either in the presence (+) or absence (-) of novobiocin. The additional 15 min represents the time required for experimental manipulation; novobiocin was also present during this period when applicable.

synthesis initiated at *oriA* and *oriB* must be present in sufficient amounts under our experimental conditions. They are either transported into the chloroplast from the cytoplasm or they are stable.

#### A novobiocin-insensitive mode of chloroplast DNA synthesis is initiated in the IRs after a delay

Surprisingly, when algal cells were pulse-labeled with  $^{32}\text{P}$  at 8 or 24 hr after addition of novobiocin, some chloroplast DNA synthesis had resumed. However, only a subset of chloroplast *Hpa* II restriction fragments was labeled under these conditions (Fig. 2, D and E). It is important to note that superhelical tension in chloroplast DNA at these times is still low, as measured by

HMT intercalation *in vivo* (25, and additional unpublished results).

After 8 hr of novobiocin treatment, chloroplast *Hpa* II fragments 1, 2, the 8/9 doublet, 17, and the 18/19 doublet were selectively labeled (Fig. 2D). After 24 hr, these same restriction fragments as well as *Hpa* II fragments 5 and 7 were labeled (Fig. 2E). Note that all these labeled *Hpa* II restriction fragments are located in only one of the two SCRs (see Fig. 1A). *Hpa* II fragments 3, 4, 6, and 10 in the other SCR were not labeled, nor were fragments 14/15 which are located in the IRs (Fig. 1A).

The selective labeling of chloroplast DNA under these conditions was due to replication, not repair synthesis. The same *Hpa* II chloroplast restriction fragments which were preferentially labeled under these conditions preferentially accumulated in cells grown in the presence of novobiocin for 48 hr as measured by ethidium-stained agarose gels of unlabeled, total DNA (data not shown).

The labeling pattern of *Hpa* II restriction fragments indicates that novobiocin-insensitive DNA synthesis is initiated in regions of the chromosome different from the two origins of replication, *oriA* or *oriB* (see Fig. 1A) (2). For example, *Hpa* II fragments 5 and 7, which are closer to these origins than *Hpa* II fragments 1 and 2 (Fig. 1A), were labeled later than fragments 1 and 2. Since there was little or no DNA synthesis in the opposite SCR, most replication forks would have to traverse *Hpa* II fragments 5 and 7 before entering fragments 1 and 2 if they had been initiated at *oriA* and/or *oriB*.

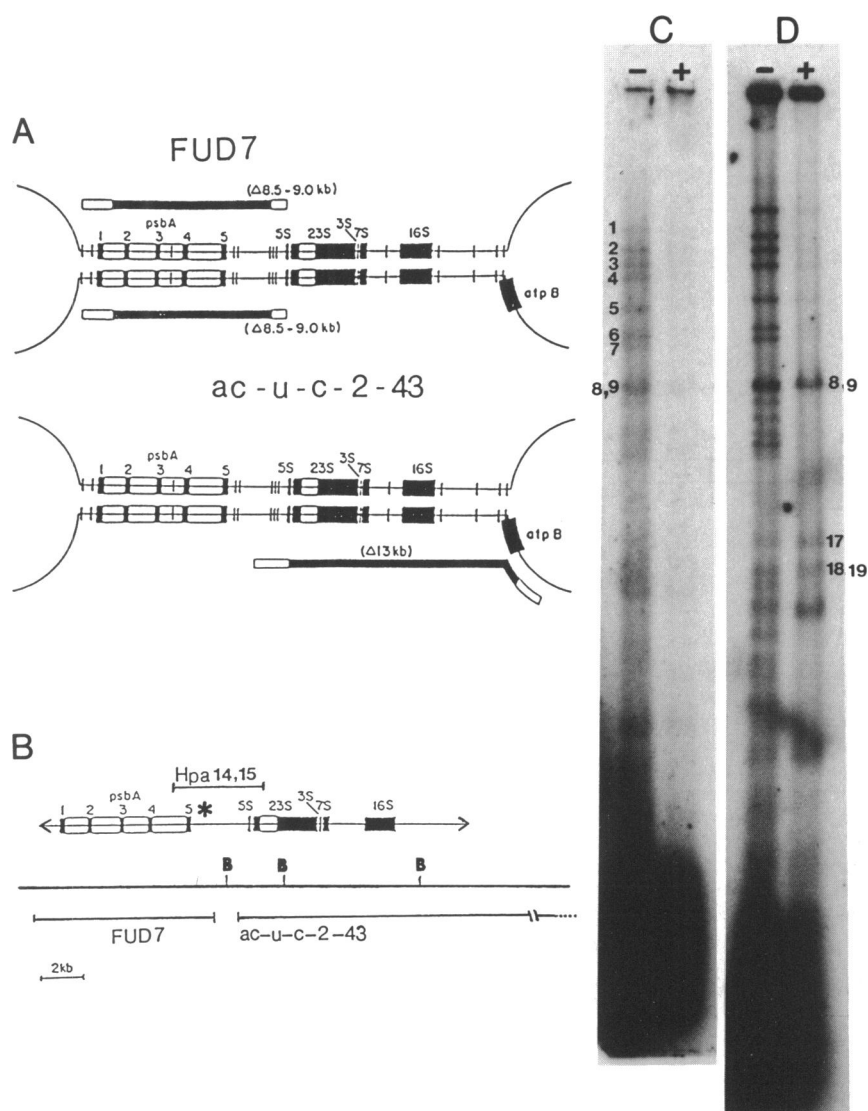
Total algal DNA from cells exposed to novobiocin and kanamycin for 48 hr displayed the same altered chloroplast *Hpa* II restriction pattern as total DNA from cells treated with novobiocin alone (data not shown). Chloroplast protein synthesis is not necessary for normal chloroplast DNA replication, nor for the novobiocin-insensitive bypass mode. Any required proteins must be imported into the chloroplast.

#### DNA sequences in the IRs are required for novobiocin-insensitive chloroplast DNA replication

The temporal and spatial pattern of DNA synthesis suggested that novobiocin-insensitive DNA replication is initiated in the IR regions in or near *Hpa* II fragments 14/15, that it is unidirectional under these conditions and that it stalls or stops near *Hpa* II fragments 5 and 7 (see Discussion). To test this possibility, novobiocin-insensitive chloroplast DNA replication was examined in two mutant strains in which different regions of the IR are deleted (Fig. 3A). Note that in both of these strains, deletions extend into the *Hpa* II 14/15 region of the IR (Fig. 3B).

The strain FUD7 lacks an 8.5–9 kb segment of the *psbA* region in both copies of the IR (Fig. 3A) (34, 35). In contrast, the strain *ac-u-c-2-43* lacks the ribosomal RNA cistrons of only one of the IRs and the adjacent *atpB* region (Fig. 3A) (34, 36).

When FUD7 cells were grown with novobiocin for 24 hr and then labeled with  $^{32}\text{P}$  under conditions identical to those used for wild-type cells, little or no  $^{32}\text{P}$  was incorporated into any of the chloroplast *Hpa* II restriction fragments (Fig. 3C). As expected, there was little if any accumulation of chloroplast DNA in FUD7 cells treated with novobiocin for 48 hr (i.e., the chloroplast DNA copy number was greatly reduced)(data not shown). In contrast, all chloroplast *Hpa* II restriction fragments were labeled in untreated cultures of FUD7 cells (Fig. 3C). Like in wild-type cells, nuclear DNA synthesis in this strain was relatively unaffected by novobiocin. Together these results show that the



**Figure 3.** Deletion of IR DNA sequences affects novobiocin-insensitive chloroplast DNA synthesis. **A.** Maps of the IR DNA sequences deleted in the strains FUD7 (symmetric deletions) and *ac-u-c-2-43* (asymmetric deletion); the sequences deleted are indicated by bars (the open ends denote uncertainty in the endpoints of the deletions) (from ref. 34). **B.** A map of the IR region including *Hpa* II 14/15. An asterisk indicates the approximate location of a hotspot for recombination (27). The positions of *Bam* HI sites are shown below. The IR DNA sequences deleted in FUD7 and *ac-u-c-2-43* cells are shown on the lowest line. **C.** Total DNA, labeled *in vivo* with  $^{32}\text{P}$ , was prepared from FUD7 cells grown in the presence (+) or absence (-) of 450  $\mu\text{g/ml}$  novobiocin for 24 hr. Chloroplast *Hpa* II fragments are numbered as in previous figures. **D.** Total DNA, labeled *in vivo* with  $^{32}\text{P}$ , was prepared from *ac-u-c-2-43* cells grown in the presence (+) or absence (-) of 450  $\mu\text{g/ml}$  novobiocin for 24 hr. Chloroplast *Hpa* II fragments synthesized in the presence of novobiocin are indicated.

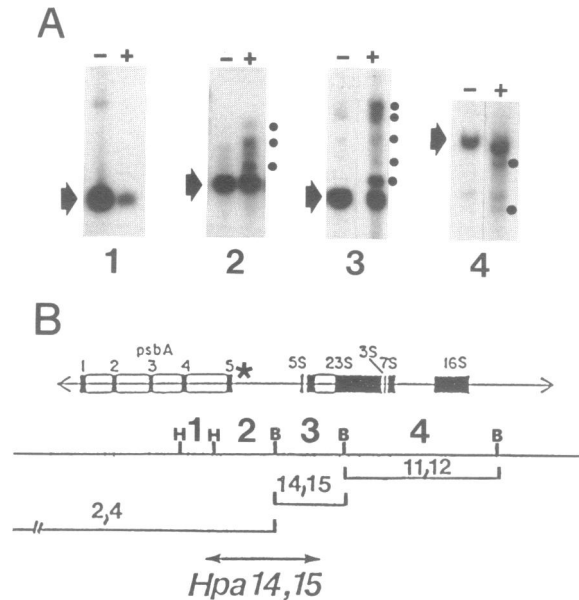
deletions in FUD7 cells specifically inhibit the novobiocin-insensitive mode of replication.

When *ac-u-c-2-43* cells were treated with novobiocin for 24 hr and labeled with  $^{32}\text{P}$ , novobiocin-insensitive DNA synthesis occurred but the labeling pattern was different from that of wild-type cells. Only three chloroplast *Hpa* II restriction fragments were labeled: the *Hpa* II 8/9 doublet, *Hpa* II 17, and the *Hpa* II 18/19 doublet (Fig. 3D). In contrast to wild-type cells, *Hpa* II fragments 1, 2, 5, and 7 were not labeled. These results suggest that DNA synthesis is initiated only in the IR without the deletion, and that replication forks proceed unidirectionally into the SCR toward *Hpa* II 5 where they stall. Like in FUD7 cells, in untreated *ac-u-c-2-43* cells all chloroplast restriction fragments were labeled (Fig. 3D).

Together, the results with the FUD7 and *ac-u-c-2-43* strains further suggest that certain IR DNA sequences in the *Hpa* II 14/15 region are specifically required for novobiocin-insensitive chloroplast DNA replication.

#### Novobiocin-insensitive chloroplast DNA replication in the IRs

The results described above imply that there is a transition region in the IRs between replicated and unreplicated segments of the chromosome. This should be reflected by the extent of replication and perhaps by the presence of unusual DNA structures in this transition region. We tested these implications by probing Southern blots of restriction digests of unlabeled total DNA from cells treated with novobiocin and from untreated cells, with different labeled chloroplast restriction fragments spanning the



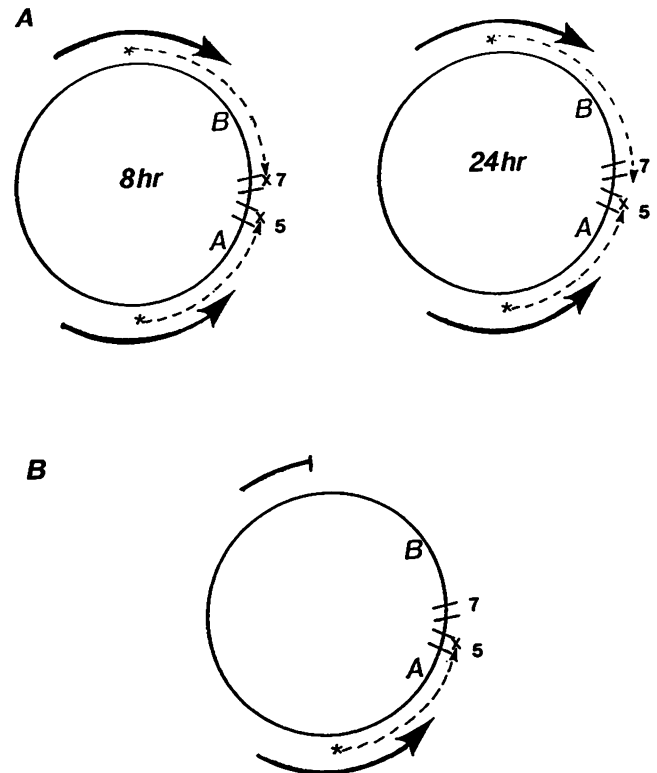
**Figure 4.** Southern blots of total DNA probed with DNA fragments from different positions on the chloroplast genome. Unlabeled total DNA was prepared from *cc278 cw-15* cells grown either in the presence (+) or absence (-) of 450  $\mu\text{g/ml}$  novobiocin for 48 hr. Aliquots of these DNA samples were digested with restriction enzymes, fractionated by electrophoresis and blotted to nitrocellulose. Each Southern blot was probed with a different cloned chloroplast restriction fragment. **A.** Lane 1, *Hind* III digest/probe 1; lane 2, *Hind* III-*Bam* HI digest/probe 2; lane 3, *Bam* HI digest/probe 3; lane 4, *Bam* HI digest/probe 5. The arrowheads indicate restriction fragments of expected mobility. Novobiocin-induced restriction fragments which migrate anomalously are indicated by dots. **B.** A map of the IR region (as in Fig. 3); the positions of probes 1-4 are indicated. The location of a hotspot of recombination (27) is indicated by an \* and the position of *Hpa* II fragment 14 (or 15 in the opposite IR) is shown. A restriction map of the IR region is shown below; B=*Bam* HI, H=*Hind* III. Only the relevant *Hind* III sites in the IR are shown.

putative transition region or with fragments from both SCRs as controls.

In the IR region, the relative abundance of *Hind* III fragment I (Fig. 4B) was greatly reduced in novobiocin treated cells (Fig. 4A, probe 1). In contrast, the abundance of the adjacent *Hind* III-*Bam* HI fragment 2 (Fig. 4B) was reduced very little (Fig. 4A, probe 2). These results suggest that the transition region is in or near *Hind* III-*Bam* HI fragment 2, which overlaps the *Hpa* II 14/15 region (Fig. 4B).

Together with the expected restriction fragments, additional novobiocin-induced DNA fragments were detected by probes 2, 3 and 4 (Fig. 4A and B). In contrast, such novobiocin-induced fragments were not detected in four different regions at considerable distances from the transition region (two regions in each SCR) (data not shown). In all these experiments, DNA samples from control and novobiocin-treated cells were used to prepare several Southern blots, each of which was hybridized with one of the above probes under otherwise identical conditions.

The additional bands are most likely not due to cross-hybridization with multiple, short repeated sequences which are found throughout this region of the IRs (34). Cross-hybridization fails to explain the additional bands that are induced by novobiocin. We suggest that the additional bands detected with probes 2, 3 and 4 represent anomalously-migrating structures. Anomalously-migrating fragments corresponding to probes 2 and



**Figure 5.** An interpretation of novobiocin-insensitive DNA replication. Novobiocin-insensitive DNA replication is initiated from a preferred region of the IRs (indicated by an \*) and proceeds unidirectionally into the SCR containing *Hpa* II fragments 5 and 7. **A.** In wild-type cells treated with novobiocin, replication forks enter the replicated SCR from both IR regions with equal probability since each IR contains the preferred initiation region. Eight hours after the addition of novobiocin, replication forks moving in the A to B direction terminate (X) near or in *Hpa* II 5 and forks moving in the B to A direction pause or terminate near *Hpa* II 7. Twenty-four hours after the addition of the drug, replication forks moving in the A to B direction terminate in or near *Hpa* II 5 (X). Forks moving in the opposite direction (B to A) no longer pause or terminate in the *Hpa* II 7 region, but instead progress through this area of the chromosome. In FUD7 cells, an essential part of the preferred initiation region is deleted, thus novobiocin-insensitive DNA replication is not initiated. **B.** In *ac-u-c-2-43*, cells a second essential part of this region is deleted. Therefore, in cells treated with novobiocin, replication forks enter the replicated SCR from only one IR (i.e., the IR which contains the intact initiation region, \*) and terminate (X) near *Hpa* II 5.

3 were also observed in cells not treated with novobiocin, albeit at greatly reduced proportions when compared with novobiocin-treated cells (Fig.4A).

## DISCUSSION

Our results show that:

- i) Novobiocin rapidly inhibits overall chloroplast DNA synthesis *in vivo* (within ~45 min after treatment).
- ii) After prolonged exposure to the drug (~8 hr), a novobiocin-insensitive bypass mode of DNA synthesis becomes operative.
- iii) Novobiocin-insensitive DNA synthesis follows different temporal and spatial patterns from chloroplast DNA replication without novobiocin. In contrast to replication initiated at *oriA* and *oriB*, novobiocin-insensitive DNA synthesis requires DNA sequences in the IRs and results in only partial replication of the chromosome.

iv) Inhibition of chloroplast protein synthesis does not affect novobiocin-insensitive DNA synthesis.

v) Anomalous-migrating DNA fragments specifically accumulate in or near the preferred initiation region for novobiocin-insensitive DNA replication.

We conclude that novobiocin, like in bacteria, inhibits DNA replication from the known chloroplast origins, *oriA* and *oriB*. Subsequently, novobiocin facilitates or uncovers another mode of DNA replication which is initiated predominantly within the large IR regions. We now discuss separately the effects of novobiocin on these two modes of DNA replication in terms of the inhibition of one or more chloroplast type II topoisomerases. At the lowest concentrations of novobiocin that we used here (250  $\mu\text{g/ml}$ ), these enzymes are selectively inhibited by novobiocin (25, see Introduction) and the average superhelical tension of chloroplast chromosomes is reduced.

This inhibition of DNA synthesis is readily explained as a consequence of the topoisomerase inhibition. Initiation from the two origins, *oriA* and *oriB* *in vitro* is enhanced by supercoiling of the template DNA (3). It is plausible to assume that supercoiling would be required for assembly of an initiation complex and for 'open-complex' formation at the chloroplast origins *in vivo*, as it is required in many other systems (for review, see 15). In addition, it is possible that transcription from the promoters in one or both of these origins (3, 37) is required to open the double helix or to synthesize primers, or both, and that novobiocin affects this transcription. This drug has been shown to differentially affect synthesis of chloroplast transcripts (10, 25, 38).

Inhibiting initiation at *oriA* and *oriB* can only partially explain the rapid inhibition of overall chloroplast DNA synthesis by novobiocin. The rate of fork movement in *Chlamydomonas* chloroplasts is estimated at  $\sim 15-20$  bp/sec (39). At this rate, each chloroplast replisome is predicted to synthesize  $\sim 30$  kb of DNA during the labeling period used here. The replication forks initiated prior to addition of novobiocin are expected to be still active during the earliest labeling period that we could employ ( $\sim 45$  min). The severe inhibition of chloroplast DNA synthesis at the earliest labeling period suggests that existing replication forks slow or terminate when chloroplast type II topoisomerases are inhibited with novobiocin. We do not know whether novobiocin-sensitive topoisomerases are also necessary for resolution of replicated chloroplast DNA molecules.

The resumption of chloroplast DNA replication after prolonged exposure to novobiocin was at first unexpected. This replication occurs in spite of the persistent inhibition of chloroplast type II topoisomerases and the persistent low level of superhelical tension in chloroplast DNA (25). We surmise that novobiocin-insensitive replication forks are different and are initiated by a different mechanism than those initiated at *oriA* and *oriB*. This mechanism most likely involves recombination.

The temporal and spatial patterns of novobiocin-insensitive DNA replication and deletion analysis suggest that most replication forks are initiated in the IRs, in or near *Hpa* II fragments 14/15. We have considered two plausible scenarios for initiation of novobiocin-insensitive DNA replication:

a) A cryptic origin in this region is activated or uncovered when chloroplast type II topoisomerases are inhibited.

b) There is a hotspot of recombination near this region and novobiocin-insensitive DNA replication is initiated from intermediates of recombination (40, 41).

In either case, replication is postulated to be unidirectional from the preferred initiation region and to pause or terminate near the *Hpa* II 5 and 7 regions when novobiocin inhibits the topoisomerases (Fig 5). Because the preferred initiation region is located in the IRs, replication forks can be initiated in either one or the other IR with equal probability (Fig. 5A). Because the initiation regions are inverted relative to each other in the two IRs, only one of the SCRs is replicated. To explain the pattern of DNA synthesis in the deletion mutants, we propose that in FUD7 cells an essential cis-acting element of the preferred initiation region is deleted; therefore, novobiocin-insensitive DNA replication is greatly reduced. In *ac-u-c-2-43* cells, a different essential cis-acting element is deleted in one IR, thus replication forks can enter the replicated SCR from only one direction (Fig. 5B). We postulate that these replication forks terminate near the *Hpa* II 5 region of the chromosome. These DNA sequences may act as termination signals (possibly due to protein binding) or they are difficult to replicate when superhelical tension in the chromosome is reduced. We do not know whether replication forks terminate near *Hpa* II 5 (or other regions) in the absence of novobiocin.

The cryptic origin hypothesis (a) is based on the knowledge that in peas two major chloroplast replication origins are located near the ribosomal RNA genes which are homologous in all chloroplasts (42, 43). Chloroplast chromosomes in *Chlamydomonas* may contain relatives of these origins, but replication from *oriA* and *oriB* overshadows initiation from these origins under normal laboratory conditions. This hypothesis offers no explanation why initiation of novobiocin-insensitive replication requires prolonged growth in novobiocin. Furthermore, the FUD7 deletions which inactivate this bypass replication mode would not eliminate these potential pea-like origins.

In support of the recombination mechanism (b), a hotspot of recombination has been mapped in the IR near the 3' end of *psbA* (27) and this region is deleted in the mutant FUD7. One version of the recombination-dependent mechanism is based on the double-strand break repair model (44, 45) combined with initiation of replication from recombination intermediates (40). In this version of the model, an initial double-strand break would be close to, but not necessarily at the initiation site of DNA replication. Branched recombination intermediates can be converted to replication forks at several (different) distances from the sites where recombinational invasions or pairings were initiated (41, 46). One or more preferred conversion sites might be deleted in the *ac-u-c-2-43* mutant, although there are other possible explanations why novobiocin-insensitive replication is defective in this mutant.

In the context of the recombination model, it is interesting that several anomalously-migrating restriction fragments accumulate specifically in the IRs in a region to the right of the transition between replicated and non-replicated DNA segments, but not in any other region of the chloroplast chromosome, after novobiocin-treatment. It is tempting to speculate that some or all of these anomalous fragments represent intermediates in the novobiocin-insensitive initiation mechanism. The structure of the anomalously-migrating restriction fragments and the structure of the partially-replicated chloroplast chromosomes requires further investigation. We believe that the partially-replicated chloroplast chromosomes in novobiocin-treated cells are either Y-shaped DNA molecules, or  $\alpha$ -shaped molecules which still contain branched recombinational intermediates,



Within the context of the recombination model, the delayed onset of novobiocin-insensitive DNA replication suggests that recombination proteins have to accumulate before the mechanism becomes effective. This situation is similar to inducible stable DNA replication (iSDR) of *E. coli* which can operate when the chromosomal origin, *oriC*, is not functioning (47, 48). In *E. coli*, this unusual DNA replication requires accumulation of active RecA protein (49, 50). It is thought to be initiated from recombinational intermediates. In this respect, it is noteworthy that a nuclear-encoded RecA-like protein accumulates after novobiocin treatment in *C. reinhardtii* (R. Sayre, personal communication).

We can only speculate on the importance of novobiocin-insensitive DNA replication for practical applications and for *Chlamydomonas* as an organism. This mechanism is not essential for *Chlamydomonas* under laboratory conditions, for example, FUD7 mutants are viable. However, initiation in the IRs may augment origin initiation and may become important under certain growth conditions, specifically those which lead to differences in superhelical tension of chloroplast chromosomes without necessarily inactivating type II topoisomerases (25). We suggest the possibility that this replication mechanism contributes to the recovery of copy number of chloroplast chromosomes after depletion by 5-fluorodeoxyuridine (FUD) (51) or novobiocin (R. Sayre, personal communication), and the success of chloroplast transformation after such treatments (52). Under these conditions when recovery occurs in the absence of drugs, initiation from the large inverted repeats may be bidirectional and entire chloroplast chromosomes may be replicated.

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