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Regulation of chloroplast DNA replication in *Chlamydomonas reinhardtii*[1]

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Abstract. The initiation step of a DNA replication cycle is the prime target for the regulation of DNA replication. The switch that governs this process and how it is influenced by other biosynthetic and metabolic processes is a major issue in biology. The controlled nature of chloroplast (cp) DNA replication and the relatively less complex and manipulatable metabolic environment of the organelle qualify the cp DNA replication system for the study of regulation. To understand the molecular mechanism of regulation, it is important to identify the biological switch and the motifs used by the switch. Toward this end, one cp DNA replication origin in *C. reinhardtii,* OriA, was mapped and cloned. A membrane-associated protein which bound strongly with cloned OriA *in vitro* was identified as *frx* B, an iron-sulfur redox protein subunit of cp NADH dehydrogenase. A sequence-directed bent DNA was detected within OriA. A 224 bp HgiAI fragment subclone with the bending locus positioned in the middle was necessary and sufficient to display replication function. In this study, detailed interaction between *frx*B and OriA *in vitro* and *in vivo,* and the developmental changes of cellular *frx*B distribution were investigated. The cp NADH dehydrogenase complex is rather closely related to the mitochondrial complex I except that the cp enzyme prefers plastoquinone (PQ) as its electron acceptor. In cp, the redox state of PQ pool has a central role in regulation. The current data support the following hypothesis. The membrane attached complex is the site of DNA replication in vivo and it is also the structure through which control factors may act. The redox state of *frx*B as well as the redox state of NADH

the detailed mechanism has proven difficult to study (Baker *et al.,* 1988). We are using the chloroplast (cp) DNA replication system in a well studied eukaryotic alga, *Chlamydomonas reinhardtii,* to investigate this fundamental question. Cp contain multiple copies of a relatively simple circular genome (Rochaix, 1978; Palmer, 1986). Through the use of the DNA-specific fluorochrome, 4',6-diamidino-2-phenylindole (DAPI), cp nucleoids were seen as pleomorphic regions of condensed DNA in *Chlamydomonas* (Coleman, 1978). Moreover, the cp nucleoids change dynamically during different developmental stages (Kuroiwa *et al.,* 1981, 1982; Coleman, 1984; Nakamura *et al.,* 1986; Ehara *et al.,* 1990). As judged by quantitative fluorescence measurements, newly released zoospores contain 3.5 times as much cp DNA as gametes which were induced and maintained in nitrogen starvation condition (Coleman, 1984). Cp genome copies increase transiently from 85 to 340 within the single cp in *Chlamydomonas reinhardtii* when non-dividing cells showing almost no oxygen -evolving activity are shifted onto fresh medium (Nakamura *et al.,* 1986). In higher plants, the dramatic per -cell increase in cp genome copy number that accom-

sary and sufficient to display replication function in the *in vitro* DNA replication system (Hsieh *et al.,* 1991). In this report, we further investigate the sequence specific protein-DNA interactions in the Ori A region and the dynamic changes of *frx*B distribution at different algal developmental stages. We also discuss the possible implication of these experimental data to the regulatory mechanism of cp DNA replication.

Materials and Methods

Algal Strains and Culture Conditions

Chlamydomonas reinhardtii CC124 (mt^-) and CC125 (mt^+) and CC278 (CW-15 mt^-) were obtained from *Chlamydomonas* Genetics Center (Department of Botany, Duke University, Durham, North Carolina, USA). Phototropic cultures were synchronized by growth in Sueoka High-Salt Medium (HSM) on a 12 h. light/12 h. dark cycle according to Surzycki (1971). Treatment with 5-fluorodeoxyuridine (FdUrd) was the same as Matagne & Hermesse (1981). Gametogenesis of cells harvested midway through the light period in nitrogen-free medium and mating were performed

were reported previously (Nie *et al.*, 1987; Wu *et al.*, 1989). IgG fraction was purified by using the Affi-Gel Protein A MAPS II kit (Bio-Rad). Protein concentration was determined by using Bio-Rad protein assays.

Band Shift DNA-Binding Assay

Band shift assay using high ionic strength polyacrylamide gel electrophoresis was performed essentially according to Staudt *et al.* (1986). Restriction sites used to generate various restriction fragments for this assay are shown in Fig. 1. Each gel-purified restriction fragment was either labelled at the 3' end by using the Klenow fragment of *E. coli* DNA polymerase I (BioLab) or at the 5' end by using the T_4 polynucleotide kinase (BRL) (Maniatis *et al.*, 1982). For each batch of E3 protein and end-labelled fragment, titrations were conducted to determine the optimal concentration of poly (dI·dC)·poly(dI·dC) (Sigma) used to compete for nonspecific binding sites and the saturation level of E3 protein. The binding buffer contained 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 50 μg/ml BSA. In a typical experiment, approximately 5 ng end-labelled probe DNA, 8 μg E3 protein and 1.25 to 5 μg poly(dI·dC)·poly(dI·dC) were incubated in 20 μl binding buffer at room temperature for 1 h before polyacrylamide gel electrophoresis and autoradiography. The amount and type of cold competitive DNA used in each experiment are described in the text.

DNase I Footprinting and DMS Methylation Protection Analysis

End labelled DNA fragment was prepared from pBHg. The 224 bp insert was obtained by treatment with *Hind* III/*Bam* HI and gel purification. After labelling at either 3' ends or 5' ends, fragment with single end labelled was generated by treatment with either *Eco*RI or *Sma*I respectively. Both the coding strand were carried out in the modified binding buffer in which the 50 mM Tris was replaced by 50 mM Na Cacodylate, 1 μl dimethyl sulfate (DMS) was added to 200 μl reaction mixture and the reaction was carried out at 20°C for 2 min. Methylation stop, cleavage at the methylated base with piperidine, separation on sequencing gel and autoradiography were carried out according to Maxam and Gilbert (1980).

In vitro UV Crosslinking, Isolation of Proteins Crosslinked to cp DNA, Radioiodination of Proteins and Immunoprecipitation

The wall-less strain *C. reinhardtii* CC278 was used for this experiment. Algal cultures were grown in HSM medium until early log phase or stationary phase as described in the text. At that time, 2 liters stirring algal culture in a shallow container was irradiated with UV from 4 cm directly above by a UV transilluminator (VWR 254 nm) for 30 min. The algal cells were then collected by centrifugation and treated in lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-P-40, 0.02% NaN_3 and protease inhibitors (Nie *et al.*, 1987). After shaking at rm temp for 30 min, solid CsCl and ethidium bromide (EtBr) was added. The final mixture contained 300 μg/ml EtBr with a refractive index of 1.3990. Centrifugation and collection of the DNA band and subsequent separation of cp DNA from mitochondrial DNA and nuclear DNA in a CsCl gradient containing Hoechst dye 33258 was carried out according to Aldrich *et al.* (1985). The purity of cp DNA was checked by analyzing its *Eco* RI restriction pattern.

The cp or nuclear DNA band was separately collected, dialysed against 1 mM phosphate buffer (pH 7.2) containing protease inhibitors (Nit *et al.*, 1987) and concentrated by lyophilization. Fractions containing 10 μg DNA each were subjected to radioiodination with Iodo-

ed to UV crosslinking were carried out as controls. A similar attempt was also made to detect whether any protein was copurified with nuclear DNA in the UV crosslinked algal culture. Immunoprecipitation using protein A-cell suspension (Sigma P-7155) was carried out according to the procedure used by Moreland *et al.* (1985). Preimmune serum or IgG purified from preimmune serum was used in controls. In some experiments, 10 pg of BSA was added to monitor the efficiency of radioiodination reaction and immunoprecipitation reaction.

Enrichment of DNA Replication Proteins and the Complementation Test

Isolation of soluble protein from the algal cells and the protein purification procedures using the DEAD-cellulose, heparin-agarose and single-stranded DNA-agarose columns, and glycerol gradient were described in Wang *et al.* (1991). DNA polymerase peak fraction in the glycerol gradient was identified by DNA polymerase assay using activated calf thymus DNA as template (Wong *et al.*, 1991). *In vitro* DNA replication assay using supercoiled DNA of the plasmid pBHg as template (Wu *et al.*, 1986) was used to monitor the complementation activity of each glycerol fraction to the peak fraction of DNA polymerase.

Immunocytological Localization of frx B Protein and DNA Visualization

At different time points, algal cells were collected and fixed with 3.7% formaldehyde (Electron Microscopy Sciences) in phosphate buffered saline (PBS, 40 mM phosphate pH 7.2, 150 mM NaCl) for 4 min, washed twice with PBS, dropped onto an acid-cleaned glass slide, gently flattened under a siliconized cover slip by finger pressure and immediately frozen in liquid nitrogen. The cover slip was then flipped off with a razor blade and the algal cells on the slide were permeabilized by submerging in cold 100% ethanol at -20°C for 10 min with occasional agitation. The treated cells were washed with PBS containing 0.25M NaCl at room temperature for 10 min, with PBS for 5 min, treated with 0.5 mg/ml RNase in PBS for 1 h. at 37°C, washed twice with PBS and twice with PBS containing 0.5% Triton X-100 (PBST). A freshly diluted rabbit anti-*frx* B antiserum (usually 1/125 dilution) in PBST was applied to the cells and incubation was carried out at 37°C for 30 min and then at 4°C overnight. After washing 2 to 3 times in PBS and twice in PBST, a freshly diluted fluorescein-isothiocyanate (FITC) - conjugated goat-antirabbit IgG (Nordic, usually 1/125 dilution) in PBST was added and incubated at 37°C for 1 h. The slides were then washed 4 to 5 times with PBS, stained with 0.1 μg/ml DAPI in PBS at room temperature for 15 min, rinsed twice with distilled water and mounted in glycerol containing 0.1 mg/ml *p* -phenylenediamine, Observations were made with a Zeiss Photomicroscope III using a 100X objective and photographs were recorded using Kodak Ektar 125 color film.

The following control experiments were carried out. The anti-*frx* B antiserum was either replaced with preimmune antiserum, or preincubated with excess peptide-BSA conjugate which was used to raise the anti-*frx* B antibody (Wu *et al.*, 1989). The rabbit anti-*frx* B antiserum was omitted and later steps were carried out as described.

Results

Bent DNA Region Mapper Within Ori A Contains DNA Binding Sites for the 18 kD Membrane-Associated Iron-Sulfur Protein, frxB

DNA sequence study of the OriA region mapped primarily by electron microscopy (Waddell *et al.*, 1984) included the localization of a bent DNA locus and the delimitation of the minimal replication origin using the *in vitro* DNA replication system. It was found that the 1055 bp restriction fragment containing OriA had electrophoretic anomalies characteristic of bent DNA. A tandem dimer of the region was constructed. Quantitative measurement of the relative gel mobility of a set of permuted fragments was used to extrapolate the approximate position of the bent DNA segment. By analyzing the gel mobility of short, sequenced fragments of the bent DNA region, the putative bending locus was identified. Two A_4 tracts and two A_5 tracts were located in the bending locus. Oligonucleotide-directed mutagenesis was then used to disrupt the A tract or the spacing between A tracts, and the effect of site-specific mutation on electrophoretic mobility was analyzed. To assess the functional role of the bent DNA region, subclones containing the bending locus, mutated bending locus, and regions flanking the bending locus were constructed. Each subclone was used as

template in the *in vitro* DNA replication system which preferentially initiated DNA replication at OriA. A 224 bp *Hgi* AI fragment subclone with the bending locus positioned in the middle displayed the highest replication activity *in vitro*. Site-specific mutations or alterations of the A tracts resulted in decreased DNA bending and decreased DNA replication activity (Hsieh *et al.*, 1991).

In this study, band shift assays using various end labelled restriction fragments and a high salt extract of thylakoid membrane, the E3 fraction which contained a high concentration of *frx*B (Nie *et al.*, 1987), were carried out to define the specific protein binding region. The nucleoprotein complex detected was stable in the presence of 0.5M NaCl, therefore, a high ionic strength gel was routinely used. Fig. 1A shows the map position of each restriction fragment used for this assay. When each of the labelled fragments D, E, or Hg was used, displaced band relative to the unbound DNA and representing the nucleoprotein complex was observed in the presence of 1000 fold probe excess of poly (dI·dC)·(dI·dC) (Fig. 1D, E.). Adding 40 fold excess of respective unlabelled probe DNA in the reaction mixture completely abolished the labelled nucleoprotein complex while adding the same amount of unlabelled vector DNA, M13MP18RF or pBS had no effect (data not shown). These results suggested that the boundaries of protein binding region are adjacent to the *Bst*NI site and the second *Hgi*AI site. The shortest restriction fragment which displayed clear band shift in the presence of E3 protein fraction was the 224 bp *Hgi*AI fragment represented by line Hg in Fig. 1A. When 10 fold excess of cold probe DNA was added to the band shift mixture using this fragment, a band with less mobility shift appeared. After adding 40 fold excess of cold competitive DNA, shifted band was not detected (data not shown).

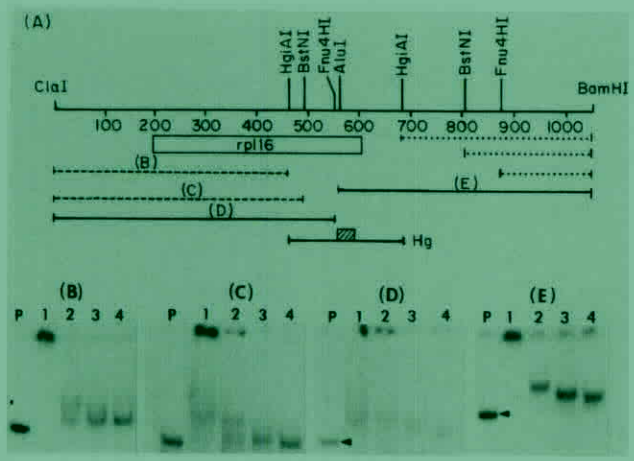


Fig. 1. Band shift experiments using different restriction fragments of the Ori A region. (A) a diagram of the Ori A region and the summary of all band shift experiments performed. The coding region of *rpl*16 gene and the restriction sites used to generate each restriction fragment are shown. Solid lines represent restriction fragments which displayed positive band shifts, broken lines represent restriction fragments with no band shifts under the experimental condition. Hg represents the *Hgi*AI restriction fragment, the stippled box shows the approximate position of the bent DNA locus mapped previously (Hsieh *et al.*, 1991). (B) (C) (D) and (E) show the band shift pattern of each respective restriction fragment, the mobility of each probe DNA is shown in lanes P. The concentration of poly (dI·dC)·poly(dI·dC) in lanes 1,2,3 & 4 are 0, 250, 500 and 1000 fold excess of labeled probe DNA respectively. Incubation conditions are described in the materials and methods section.

To determine whether the bent DNA locus located within the 224 bp *Hgi*A1 fragment was essential for protein-binding, DNA fragment with mutated bent locus region (Hsieh *et al.*, 1991) was used for the band shift assay. Fig. 2 shows the DNA sequence in each mutant. Properly spaced A tracts responsible for the anomalous gel mobility observed in bent DNA were altered in each mutant, and consequently, the mutated sequences were less effective to serve as templates in the *in vitro* DNA replication system (Hsieh *et al.*, 1991). Mutant I+IV which displayed the weakest template activity in the *in vitro* DNA replication system was also the least effective to produce nucleoprotein complex in the band shift assay (Fig. 3). When labelled DNA of each mutant was used in the band shift assay, nucleoprotein complexes of both maximal and intermediate gel mobility shift in addition to unshifted DNA were detected (Fig. 3). Radioactivities detected among these three species varied in different mutants. We speculate that protein binding sites and/or DNA sequences facilitating conformational changes after protein-binding were altered in these mutants. The rough correlation between the template activity of each mutant with its capacity to form the nucleoprotein complex with maximal mobility shift and the overlapping of the protein binding region with the functional domain of this replication origin led us to hypothesize that this protein-DNA interaction could be

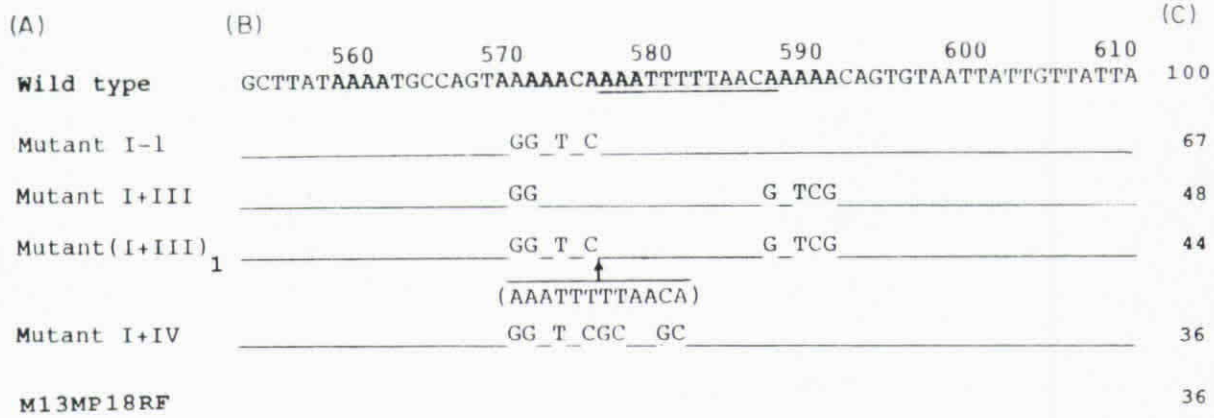


Fig. 2. DNA sequences and the relative *in vitro* DNA replication activities of various mutants which were used in the band shift experiment shown in Fig. 3.

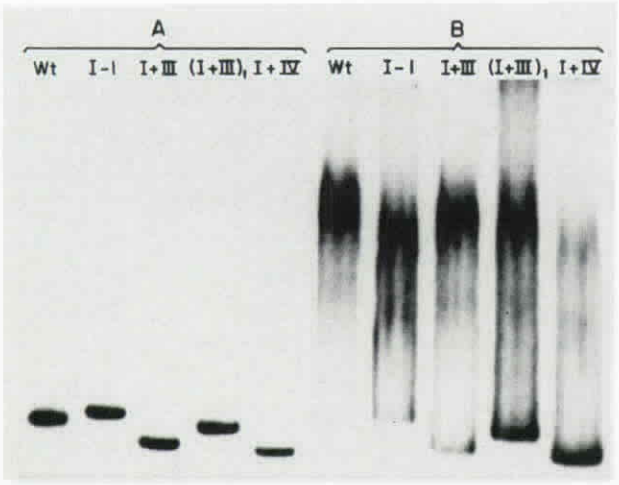


Fig. 3. Band shift experiments using mutants with altered bent locus. Saturated level of E3 fraction titrated for Wt probe which contains the original bent locus was used in every experiment shown in panel B. In panel A, one tenth of each probe used in panel B was loaded to show the relative quantity and mobility of each probe DNA in this gel electrophoretic condition.

important for cp DNA replication. Further characterization of this nucleoprotein complex was carried out.

Characterization of the Nucleoprotein Complex Containing frxB Formed in vitro

When the nucleoprotein complex containing saturated level of protein was subjected to DNase I digestion or DMS methylation, intricate but reproducible patterns were detected (Fig. 4). Data collected from

Fig. 4. The DNase I footprinting pattern and DMS methylation pattern of the coding strand of *rpl*16. Lanes C show the pattern generated in the single-end-labeled fragment in the omission as well as in the presence of heat denatured E3 fraction. Lanes T show the pattern generated in the presence of saturated level of E3. Arrows and arrowheads point to strong and weak enhanced sites respectively. GA ladder generated from this end labeled fragment is aligned with the pattern. Arrow along the GA ladder indicates the direction of transcription of *rpl*16. Open box represents the approximate position of the bent locus mapped previously by Hsieh *et al.* (1991).

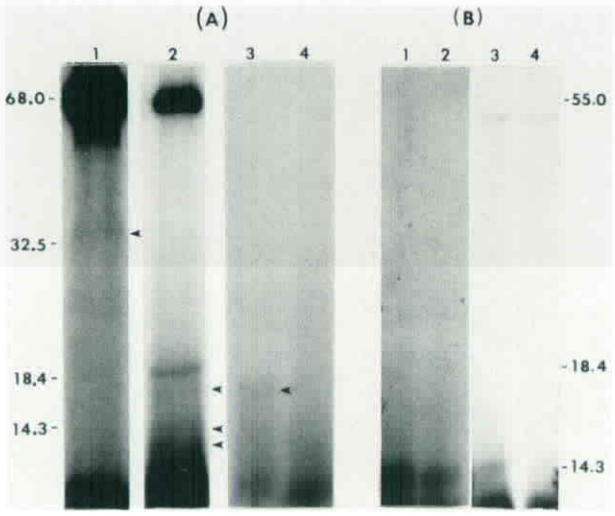


Fig. 5. Autoradiography of radioiodinated proteins that copurified with DNA in UV treated algal cells. (A) The results obtained from an early logarithmic algal culture. Lane 1 shows the proteins crosslinked with nuclear DNA. Lane 2 shows the proteins crosslinked with cp DNA. BSA was added to each preparation to monitor the radioiodination reaction. Lanes 3 and 4 show the immuniprecipitable bands when proteins identical to that shown in lane 2 but without added BSA were subjected to treatment with anti-*frx*B IgG and preimmune IgG respectively. Light bands are marked with arrowheads. Position and size of marker proteins are shown. (B) The results obtained with a stationary phase algal culture. Lane 1 & 2 show the proteins crosslinked with cpDNA. Lane 3 & 4 show the immunoprecipitation result when proteins in lane 1 were treated with anti-*frx*B IgG and preimmune IgG respectively. Position and size of marker proteins are shown.

several sets of experiment are summarized in Fig. 6. Both the DNase I footprint and the DMS methylation pattern showed characteristic repetition of enhancement region alternating with protected region. These results suggested multiple protein contact sites. Upon progressive reduction of the E3 fraction in the reaction mixture, three preferential protein-binding sites revealed (Data not shown) and a 12 bp semiconserved AT rich sequence of 5' AAAATtTTPTtT was detected in these sites. One preferential binding site overlapped with the bent center mapped previously by oligonucleotide directed mutagenesis. This sequence was demonstrated to be important for *in vitro* DNA replication (Hsieh *et al.*, 1991).

Enhancement of DNaseI cleavage or DMS methylation appeared to be especially prominent at sequences of G_{518}, A_{530}, A_{568}, $A_{581, 582}$, A_{609}, $A_{621, 622}$, A_{640}, A_{650}, and A_{662} on the bottom strand shown in Fig. 6. All methylation enhancement sites were plotted on a model of the DNA helix drawn with 10.5 bp per helical turn. Based on the rule that guanines are methylated by DMS at the N-7 position in the major groove and adenines are methylated at the N-3 position in the minor groove, the helix map showed that 36 out of the 39 methylation enhancement sites were located on one

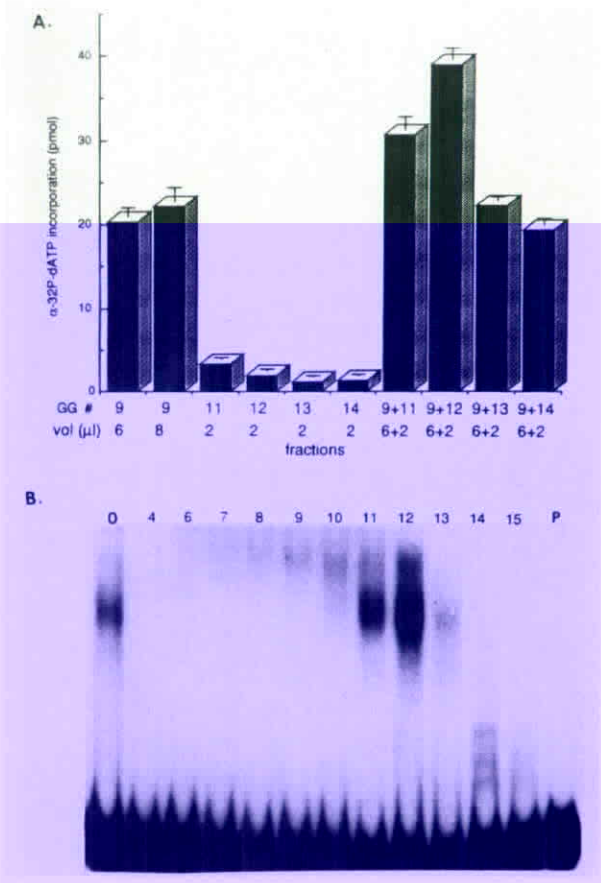
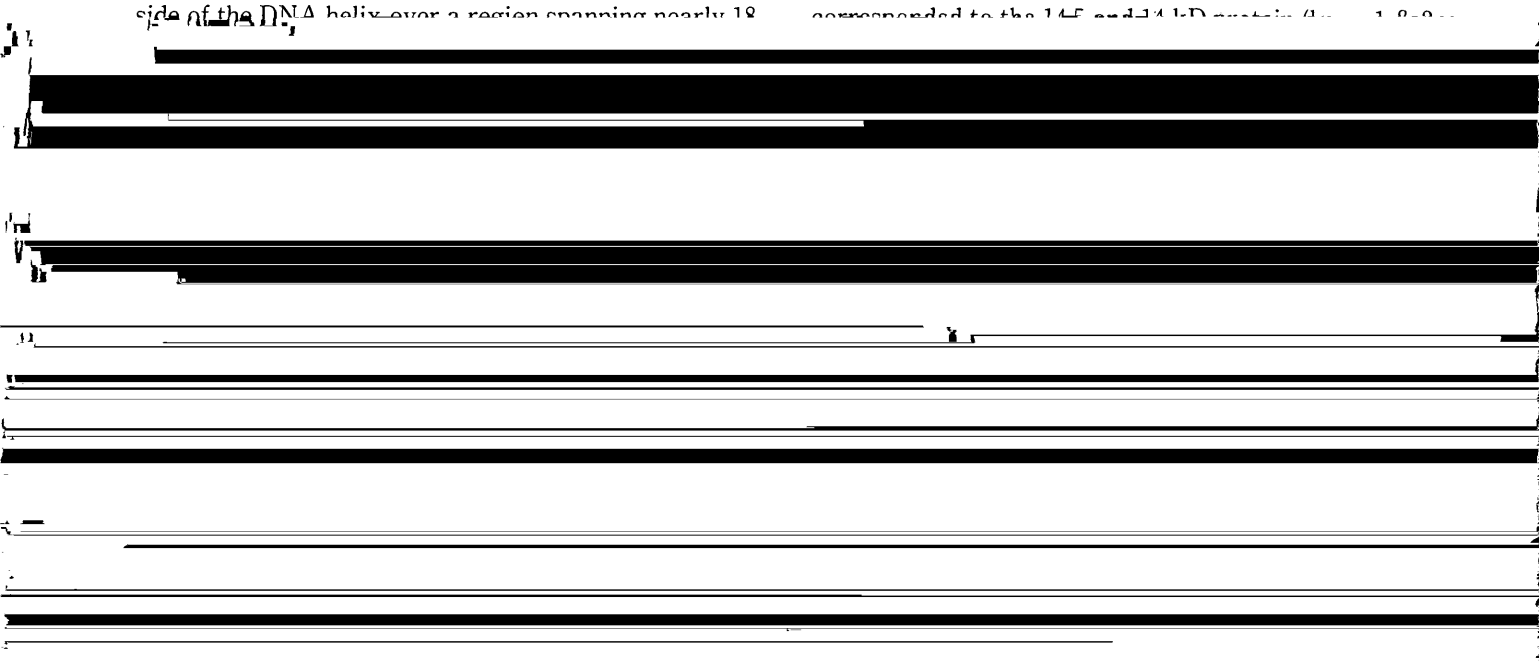


Fig. 6. (A) Results of complementation test for *in vitro* DNA replication using glycerol gradient fraction 11,12,13, and 14 to the peak fraction of DNA polymerase activity (glycerol gradient fraction 9). Each column shows the DNA replication activity of each fraction and the combination of different fractions indicated at the bottom of each column. Amount of each fraction used in the test is also shown. (B) The result of band shift experiment using each glycerol gradient fraction indicated. In lane 0, the protein mixture before the glycerol gradient separation was used. Lane P represents the probe alone without added protein.

side of the DNA helix over a region spanning nearly 18 corresponded to the 14 and 14 kD protein (



full turns. The accessibility of DNA in this long stretch to the attack of DMS suggested that it was wrapped on the outside of a large protein core. Detection of the three preferential protein-binding sites suggested that the formation of a large nucleoprotein complex might be initiated with the interaction of a few protein molecules with these binding sites.

Crosslinking of frx B Protein with cp DNA After UV Treatment of Algal Cells

To determine whether *frx*B protein interacts with cp DNA *in vivo*, algal cells used for DNA isolation were subjected to UV crosslinking before the cell breakage. After DNA purification through 2 rounds of CsCl gradient centrifugation, the AT rich cp DNA band was clearly separated from DNA bands corresponding to the mitochondrial DNA, the satellite DNA containing rRNA genes and the nuclear DNA. After collecting the cp and nuclear DNA bands separately, a fraction containing approximately 10 μg of either cp DNA or nuclear DNA was subjected to radioiodination. After the treatment, DNA was removed by DNase I and mung bean nuclease treatment and the protein was subjected to SDS polyacrylamide gel electrophoresis and autoradiography. The result obtained from cp DNA fraction isolated from an early log-phase algal culture is shown in Fig. 5A. Protein bands with apparent molecular weight of 20, 18, 14.5, 14 kD and possibly some lower molecular weight bands were detected (lane 2 of Fig. 5). In parallel experiments using fractions containing nuclear DNA isolated from the same culture, one faint band with apparent molecular weight of 33 kD and a dark band at 11 kD were detected (lane 1 of Fig. 5A). In cp DNA isolated from a control culture which was not treated with UV, iodinated protein band was not detected (data not shown). The 18 kD band was of Fig. 5B). When this protein fraction was subjected to immunoprecipitation using anti-*frx*B IgG, the crossreactivity of these bands was demonstrated, the 14.5 kD band showing a slightly stronger crossreactivity (lane 3 of Fig. 5B). Therefore, they could be structurally related to the 18 kD *frx*B protein. A previous observation from this lab also supported this spectulation. The 18 kD *frx*B protein was synthesized in cp. Treatment with chloramphenicol, an inhibitor of cp ribosomes, significantly reduced the content of 18 kD *frx*B in the E3 extract. After chloramphenicol treatment for 1 hour, the 18 kD band disappeared while the intensity of a thick band around 14 kD in that electrophoresis condition increased significantly (Nie *et al.*, 1987). The different but related UV crosslinking results obtaining from algal cultures at different growth stages also suggested the possibility of a dynamic nature in this DNA binding protein and this type of protein-DNA interaction *in vivo*.

The efficiency of UV crosslinking is usually on the order of less than 0.1% to 10% (Ausubel *et al.*, 1987). Our *in vivo* UV crosslinking efficiency was definitely on the lower side. Lane 1 of Fig. 5A shows that most histone proteins, with the possible exception of H4, were not crosslinked to nuclear DNA under this UV treatment. Therefore, the extent of interaction of *frx* B and its related proteins with cp DNA could be significant. To localize the cp DNA region containing the iodinated proteins, the radioiodinated cp DNA was treated with *Eco* RI. The resulting *Eco* RI restriction fragments were separated on a 0.8% agarose gel along with controls containing either undigested radioiodinated cp DNA preparation or *Eco* RI digest of cp DNA which was treated with proteinase K. Autoradiography showed that the restriction fragment corre-

crude *in vitro* DNA replication system (Wu *et al.*, 1986). Recently, this system has been improved substantially. This was first achieved through the purification of the DNA polymerase activity. The enzyme activity was purified by ammonium sulfate fractionation, sequential chromatographic separations on heparin -agarose, DEAE-cellulose, single-stranded DNA-agarose columns and sedimentation in a glycerol gradient. During this purification procedure, DNA polymerase activity was monitored by the incorporation of ^{3}HTTP into activited calf thymus DNA, *in vitro* DNA replication activity was measured by the incorporation of ^{32}P dATP into supercoiled plasmid DNA containing ori A. The DNA polymerase activity in its peak fraction in the glycerol gradient was purified 2160 fold, but the *in vitro* DNA replication activity in this fraction was only enriched 52 fold from the ammonium sulfate fractionated protein mixture (Wang *et al.*, 1991). The result indicated the loss of protein factors that are important for DNA replication during the purification procedure for DNA polymerase. To search for this factors(s), the *in vitro* DNA replication activity across all glycerol gradient fractions was analyzed. The peak fraction for *in vitro* DNA replication sedimented slower than the peak for DNA polymerase activity. The stimulation effect of various glycerol gradient fractions to the *in vitro* DNA replication system containing the peak fraction for DNA polymerase was measured in the complementation test. Through this test, a protein fraction which specifically stimulated DNA replication on template containing OriA was identified (Fig. 6A). Glycerol gradient fraction enriched with this putative DNA replication protein also induced specific band shift to labeled restriction fragment containing the bent locus (Fig. 6B). DNase I footprinting again revealed multiple DNA contact sites for this protein. These sites were in the vicinity of, but did not coincide with, the *frx*B binding sites. A summary of the binding sites for *frx*B and the putative replication protein is shown in Fig. 7A and 7B respectively.

Dynamic Changes of the frx B Distribution at Different Algal Developmental Stages

The immunocytological method showed that *frx*B was primarily located in vesicles adjacent to the pyrenoid in *Chlamydomonas reinhardtii*. In order to probe any possible correlation between the distribution of *frx*B protein clusters and cp DNA nucleoids within the same cell, DAPI was added to stain DNA after the intracellular *frx*B protein was decorated with FITC. Since cp DNA nucleiods undergo interesting changes at various algal developmental stages we investigated whether the cellular distribution of *frx*B showed any corresponding changes.

In *C. reinhardtii*, the most interesting and dramatic change in cp nucleoids is the disappearance of cp nucleoids from male gametes during the early stage of mating while those from the female gametes persist. This phenomenon was revealed and documented by the classic epifluorescent microscopic study of Kuroiwa *et al.* (1982). In this study, the distribution of *frx*B protein in both gametes through the mating process was monitored. In our gametogenesis condition, the gametes of both mating types were smaller than the corresponding vegetative cells. After immunocytological staining, they also appeared to contain about the same or even more *frx*B protein per cell than the vegetative cells. However, due to the absence of suitable instrumentation in our laboratory for quantitative measurement at this resolution level and the intrinsic variation of the immunocytological technique, we could not draw a firm conclusion about this observation. The cellular distribution of *frx*B in gametes was similar to that of the vegetative cells collected at 10 h after the onset of the light period (Fig. 8). This pattern was maintained in both gametes until about 30 min after mating. An example of cells collected at 10 min after mating is shown in Fig. 8A. At this stage, DAPI -stained cp nucleoids appeared unchanged in both gametes. As the mating progressed, DAPI-stained cp nucleoids in both gametes of the pair became asymmetric (Fig. 8b,c). Interestingly, *frx*B content around the pyrenoid of the male gamete which contained diminishing cp nucleoids was also reduced while that of the female gamete remained unchanged. In this experiment, the identity of male gametes was based on DAPI appearance. This unequivocal correlation was consistently observed among pairs of gametes at this stage (Fig. 8B, C). By 2 h after the mating, only one pyrenoid decorated with *frx*B was visible in the young zygote. No obvious change was evident until 12 h after the mating. At this time, the *frx*B content in the zygote reduced gradually. In mature zygotes, barely detectable *frx*B was located as one or two tiny dots around the pyrenoid (Fig. 8D). When the zygotes were incubated in the germination medium. Those *frx*B containing

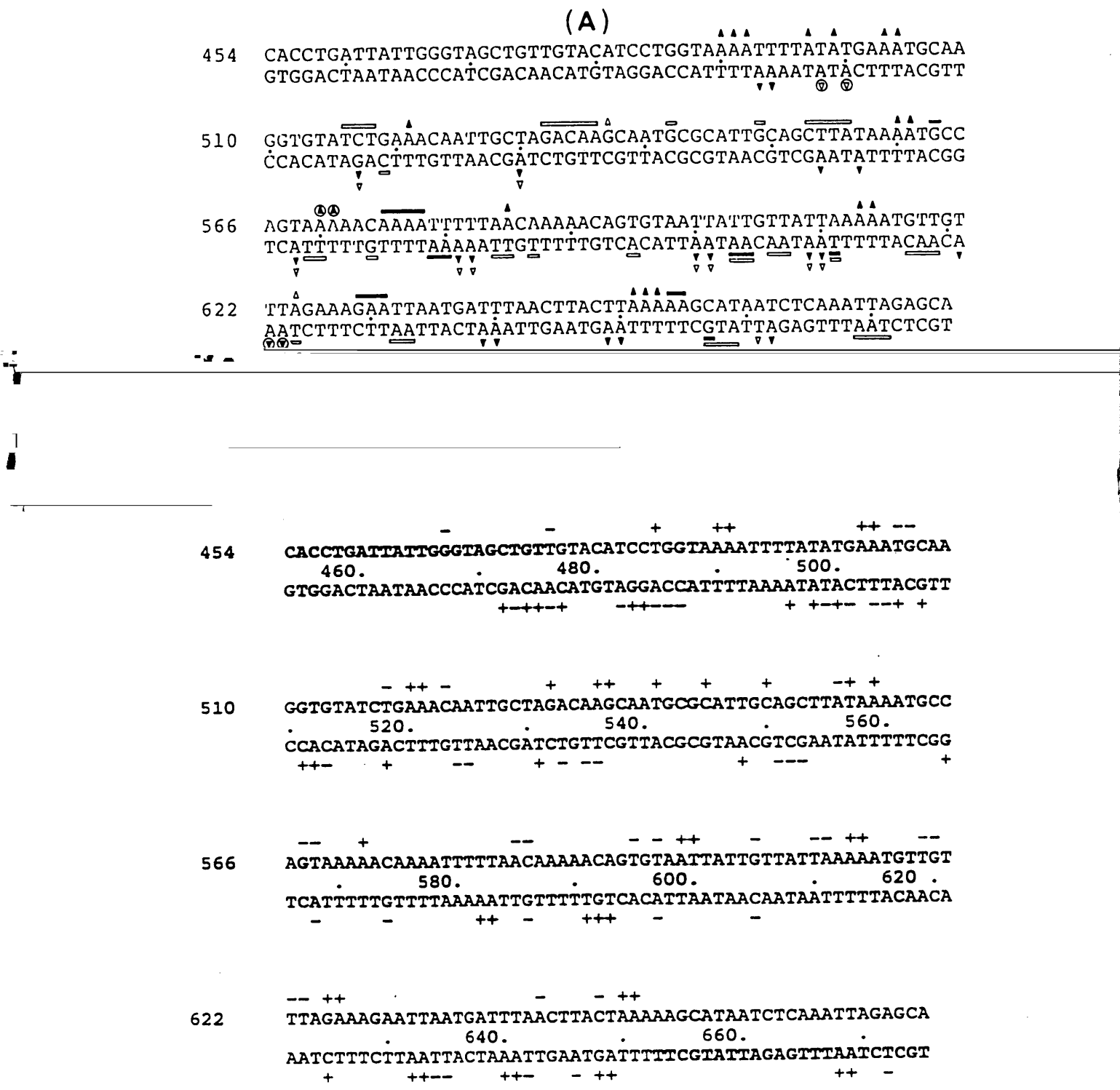


Fig. 7. (A) Summary of DNase I footprinting experiments and DMS methylation experiments. E3 fraction was added to the saturation level, all methylation enhancement (filled triangle), methylation protection (filled bar), DNase hypersensitive (open triangle) and DNase protection (open bar) sites detected are shown. Open and closed triangles enclosed within circles represent the DNase hypersensitive and methylation enhancement detected when E3 fraction was added to one fourth of the saturation level. The bottom strand is the coding strand for *rpl*16. (B) Summary of DNase I footprinting experiments using the putative DNA replication protein. +, indicates the hypersensitive site; -, indicates the protected site.

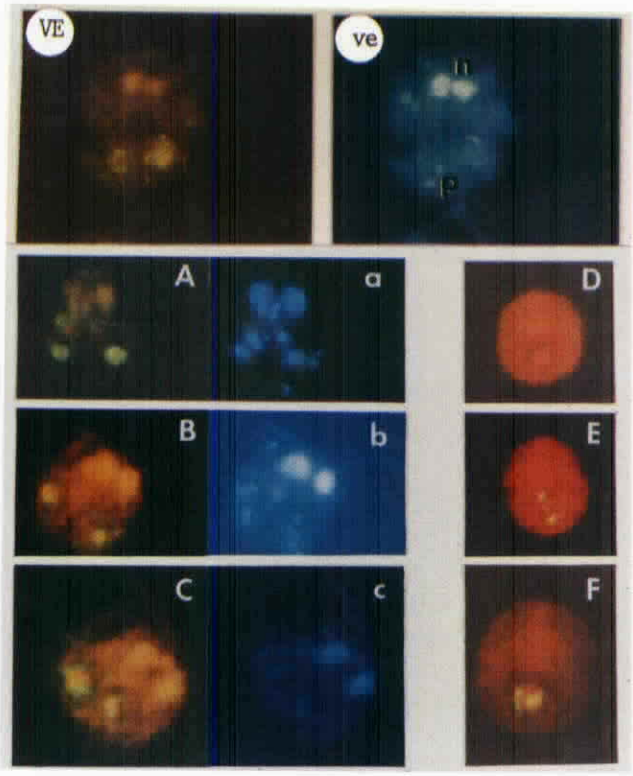


Fig. 8. Immunocytological localization of *frx*B and DAPI staining of DNA in *Chlamydomonas* cells. Fluorescent photomicrographs showing the FITC stained *frx*B (VE, A, B, C) and DAPI stained DNA of the corresponding cells (ve,a,b,c) in the vegetative cell and mating gametes respectively. The nucleus and pyrenoid in the DAPI stained vegetative cell are marked by n and p respectively. Mating gametes shown in A, a; B, b; and C, c were collected at 10, 60 and 80 min after the beginning of the mating respectively. D, E, & F show the FITC stained *frx*B in the mature zygotes treated for 0, 18 & 24 h in the germination medium respectively. The faint red fluorescence in D, E, and F was contributed by residual chlorophyll.

dots enlarged to resume the vesicle-like appearance (Fig. 8E, F). These studies again demonstrated the concomitant changes of the abundance and the distribution of *frx*B with the behavior of cp nucleoids.

Treatment with the thymidine analog, 5-fluorodeoxyuridine (FdUrd), reduced the copy number of cp DNA in *C. reinhardtii* (Wurtz *et al.*, 1977). In algal culture grown for 8 days on agar containing 1.0 mM FdUrd, a single DAPI stained cp nucleoid was detected in most cells (Matagne and Hermesse, 1981). This observation was repeated in this lab. When these cells were used for immunocytological localization, significant reduction of *frx*B protein around pyrenoid was detected. A reduced amount of *frx*B protein was detected around the pyrenoids in cells treated with FdUrd for 3 days. Only one small FITC-stained-*frx*B-containing protein dot adjacent to the pyrenoid was detected in most cells treated with FdUrd for 6 days. In *C. reinhardtii*, the condensation of dispersed cp nucleoids into one nucleoid during degeneration of the cp in aged cells was reported by Nakamura *et al.* (1986). We also repeated this observation using DAPI staining technique (data no shown). In most aged cells, we detected *frx*B protein distribution in a pattern similar to that shown in Fig. 8F. These observations also suggested that the amount of *frx*B protein around the pyrenoid was related to the cellular content and the distribution of cp DNA.

Discussion

In several DNA replication origins, organized nucleoprotein complexes formed *in vitro* have been well characterized. In most cases, the bent DNA segment in the respective replication origin facilitates the protein-DNA interaction, and the protein involved in each nucleoprotein complex is required for the initiation of DNA replication. The best studied examples are dna A protein for Ori C of *E. coli* (Bramhill and Kornberg, 1988; Yung and Kornberg, 1988), protein O for Ori λ (Dodson *et al.*, 1989), and the T antigen for SV40 Ori (Borowiec *et al.*, 1990). Formation of these nucleoprotein structures induces helix destabilization of the adjacent regions and stimulates the initiation reactions. In the Ori C-dna A protein system, the dna A protein binds specific sequences in the 245 bp OriC to form a series of complexes. ADP and ATP are tightly bound to dna A protein and are crucial to its function in DNA replication. Purified dna A protein binds to phospholipid vesicles as judged by analysis on sucrose gradient centrifugation (Sekimizu and Kornberg, 1988). Binding of various DNA replication origin to membrane has also been observed by many different groups (for review see Norris, 1990). In *B. subtilis*, origin-binding membrane-associated protein may be involved in repression of initiation of DNA replication (Laffan and Firshein, 1988). In *E. coli*, several DNA-binding membrane proteins that are specific for DNA containing Ori C have been characterized (Jacq *et al.*, 1989). Direct involvement of the membrane in the control of DNA

replication was demonstrated by the following observations. A particular membrane fraction inhibits the initiation of DNA synthesis at OriC on hemimethylated DNA templates *in vitro* (Landoulsi *et al.*, 1990). Binding of dna A protein to the head groups of acidic phospholipids in a fluid bilayer destabilizes a firmly bound nucleotide and rejuvenates the inactive ADP-form to the ATP-form. The very slow exchange of the bound nucleotide was greatly enhanced by phospholipid interaction (Sekimizu and Kornberg, 1988).

The association of cp DNA with thylakoid membrane was first reported by Woodcock and Fernandez-Moran (1968). They found that when purified spinach cp were lysed by osmotic shock, without the use of detergents, the DNA strands that commonly appeared in the electron microscope were associated with thylakoid membrane. The DNA-membrane association was also demonstrated by electron microscopic study of complete section series of young protease-treated plastid of *Beta vulgaris* L. (Herrmann and Kowallik, 1970). Both electron microscope and light microscope autoradiograph studies again confirmed the association of cp DNA with the photosynthetic membrane in spinach cp (Rose, 1979). Recently, Lindbeck and Rose (1990) reported that thylakoid-bound cp DNA from spinach was enriched for replication forks. In maize, cp DNA replication initiated within a 455 bp *Bam*HI/*Eco*RI fragment which contains a bent site (Carrillo and Bogorad, 1988). This fragment shares extensive sequence homology with our 224 bp *Hgi*AI fragment. Data presented in this report, suggested that the attachment of cp DNA at the thylakoid membrane might be facilitated by some type of interaction between Ori A and *frx* B, an iron-sulfur protein subunit of thylakoid -bound NADH dehydrogenase.

In *Chlamydomonas* and various other algae, the close association of cp nucleoids with the pyrenoid has been frequently reported (Ris and Plaut, 1962; Goodenough, 1970; Coleman, 1978, 1985; Kuroiwa *et al.*, 1981). Recently, Miyamura and Hori (1989) detected DNA in the pyrenoid matrix of a green alga, *Caulerpa okamurae*. Ehara *et al.* (1990) reported that in sychronized *Chlamydomonas reinhardtii* cells, most of the cp nucleoids gathered around the pyrenoid forming a compact mass twice during the light period: around the 4th h and the 9th h after the onset of the light period. In their culture condition, each algal cell divided to form 4 daughter cells in the 24 h growth cycle. They also suggested possible functional relations between the pyrenoid and cp-nucleoids (Ehara *et al.*, 1990). The pyrenoid of *C. reinhardtii* reproduces by fission, as does the cp itself (Goodenough, 1970). A tightly packed, dense granular material and a system of permeating tubules were detected in the pyrenoid. At the periphery of the pyrenoid, the continuity of the large pyrenoid tubule with the granum of the thylakoid system was clearly illustrated (Ohad *et al.*, 1967a,b). Based on the appearance of FITC labeled clusters and the extraction of *frx*B from thylakoid membrane fraction, we speculated that a high concentration of *frx*B could be located in this tubular region. Recently, McKay and Gibbs (1991) reported that intrapyrenoid thylakoids probably contribute little to O_2 level due to a reduction or absence of PSII activity. Therefore, this region might provide a microenvironment with a high CO_2 to O_2 ratio. There was commonly a close physical association between the pyrenoid and the polysaccharide end-product of photosynthesis (Hirschberg *et al.*, 1981). Ribulose-1, 5-bisphosphate carboxylase and nitrate reductase appear to be located primarily in the pyrenoid (Lacoste-Royal and Gibbs, 1987; Lopez-Ruiz *et al.*, 1985). Detection of glycerol-3-phosphate acyltransferase and lysophosphatidate acyltransferase in pyrenoid tubules by cytochemical technique (Jelsema *et al.*, 1982; Michaels *et al.*, 1983) suggested active membrane lipid metabolism in these tubules. Localization of *frx*B, a member of chlororespiratory electron transfer chain and a binding protein for the cp DNA replication origin, in the region might facilitate the coordination of cp DNA replication to these important cp metabolic processes. The concerted changes of *frx*B distribution with that of cp nucleoids during the various developmental processes investigated in this study support that hypothesis. Whether the change of *frx*B concentration around the pyrenoid is a cause or consequence of cp DNA replication remains to be determined.

Recently, a nucleus encoded 23 kD component of the purified bovine mitochondria complex I, or NADH -ubiquinone oxidoreductase was found to be related to *frx*B through much of its sequences. This subunit is a strong candidate for the protein that bears the N-2 redox cluster. Because of its high redox potential, N-2 is likely to be the donor to ubiquinone and its midpoint potential depends on the phospholipid content of complex I (Dupuis *et al.*, 1991). In addition to this subunit,

nuclear encoded 49 kD subunit of mitochondrial complex I was found to be homologous to the protein encoded by ORF 392 in liverwort cp genome and ORF 393 (or ndh 393) in the cp genome of tobacco and rice (Fearnley *et al.*, 1989). Therefore, cp genomes contain homologues of at least 10 components of mitochondrial complex I, with 8 of them clustered at 2 separate loci (Dupuis *et al.*, 1991). These observations suggested that the cp NADH dehydrogenase complex is rather closely related to the mitochondrial complex I except that the cp enzyme prefers plastoquinone (PQ) as its electron acceptor (Godde, 1982). The cp NADH-PQ oxidoreductase could be important for chlororespiration. The oxidation of reduced PQ by O_2 was shown in *Chlamydomonas* thylakoids (Bennoun, 1982). In this study, the immunocytological localization of *frx*B protein around the prominent subchlorplast component, pyrenoid, presented another line of evidence for chlororespiration. In the last decade, growing evidence was presented for chlororespiration in various algae and higher plants (Lin and Wu, 1990, for review see Scherer, 1990).

In cp, the redox state of the PQ pool has a central role in regulation. It is responsible for "state shift" which adjusts photosystem stoichiometry (Fujita *et al.*, 1987). It also affects the protein kinase activity in thylakoid membrane (Horton, 1983). Recently, redox regulations of protein-DNA interactions, protein-RNA interactions and the direct activation of transcription by oxidation were clearly illustrated in many studies. Several transcriptional regulatory proteins require free sulfhydryl residues for DNA binding or transcription activation (Abate *et al.*, 1990; Storz *et al.*, 1990). Fos & Jun, the protein products of the proto-oncogenes c-fox & c-jun, function cooperatively as inducible transcription factors in signal transduction processes. The dimeric protein complex interacts with DNA regulatory elements known as AP - 1 binding sites and cAMP responsive elements. DNA binding of the Fos-Jun heterodimer was modulated by redox of a single conserved cysteine residue in the DNA-binding domains. Therefore, transcriptional activity mediated by AP-1 binding factors may be regulated by a redox mecha-

enzyme activity of gene 5 protein about 100 fold and gives the enzyme a very high processivity (Tabor *et al.*, 1987). Thioredoxin plays a major role in cellular reductions in *E. coli.* It is also a hydrogen donor for ribonucleotide reductase.

In *C. reinhardtii,* reducing equivalents generated during the degradation of starch enter the thylakoidal chain at the PQ site catalyzed by NADH-PQ oxidoreductase (Gfeller and Gibbs, 1985). When the algal cells were grown under nitrogen limitation, pronounced chlororespiratory activity developed together with an altered thylakoid composition (Peltier and Schmidt, 1991). In *Chlamydomonas,* the chlororespiration rate was estimated to be around 12% to 20% of total respiration (Peltier *et al.*, 1987; Bennoun, 1982). It has been suggested that the chlororespiratory pathway provide the cell with the option of disposing of electrons formed during the degradation of starch to glycerate-3-phosphate by transferring them to various terminal electron acceptors including O_2, nitrite, protons and sulfate (Klein *et al.*, 1983; Gfeller and Gibbs, 1985). In higher-plant cells, evidence for respiratory control of photosynthetic electron transport was documented, and this control might depend on the developmental stage of leaves (Garab *et al.*, 1989). Evidences for the expression of cp ndh genes in higher plants were reported (Matsubayashi *et al.*, 1987; Lin and Wu, 1990).

According to the deduced amino acid sequence & the EPR spectrum, the 18 kD *frx*B protein contains two 4Fe-4S clusters (Wu *et al.*, 1989). The range of reduction potentials exhibited by 4Fe-4S clusters could be over 800 mV, an astonishingly large figure. An even wider range of potentials is observed for membrane -bound proteins. There are two aspects of polypeptide control of the potential: selection of the functioning pair of cluster oxidation states, and variation of the cluster potential between the selected oxidation states. In general, the polypeptide chain restricted a 4Fe-4S cluster to two of the three possible oxidation states. The barrier to the inaccessible oxidation state could be kinetic and/or thermodynamic. Some $2(4Fe\text{-}4S)^{2+;1}$ ferredoxin underwent a conformation change on reduc-

necting regulatory networks. Metabolic processes induced by nitrogen starvation or cellular responses brought about by energy requirement confer specific redox and/or conformational changes of *frx*B protein in the cp NADH-PQ oxidoreductase and consequently affect the formation and/or the type of nucleoprotein complex containing Ori A. This protein-DNA interaction could regulate the accessibility of Ori A to other

DNA replication proteins and/or modulate the active form of the origin-replication protein complex. The concerted effort of the correct topological conformation of the origin nucleoprotein complex and the availability of other DNA replication components will initiate a cp DNA replication cycle *in vivo*. Obviously, there is still much to be learned.

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衣藻葉綠體 DNA 複製之調控

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DNA 複製之主要調控關鍵在其起始步驟，此調控關鍵與其他新陳代謝之協調是目前生物界研究之一大主題。衣藻葉綠體 DNA 之複製與其生長環境有明顯之關聯。可以作爲這方面研究之範例。我們曾確定了衣藻葉綠體 DNA 之複製起點，Ori A，含有 Ori A 的限制內切酶片段已被選殖(cloned)，此一片段中含有彎曲 DNA 之 224 bp 之選殖系，就具有複製起點之功能。也發現了幾個從類囊體膜抽出的蛋白質與 Ori A 有高度親和性。其中之一已被確定爲 NADH 脫氫酶的鐵硫蛋白亞基，我們觀察了在試管中及在細胞內，此一鐵硫蛋白與 Ori A 的相互作用，也追蹤了此一鐵硫蛋白及葉綠體 DNA 在細胞中之分佈，葉綠體的 NADH 脫氫酶以質體醌(plastoquinone)爲其電子受體。在葉綠體中，質體醌的氧化還原狀況極具調控性。目前的推論是 DNA 複製所必需之多種蛋白質及 NADH 脫氫酶都能與 Ori A 形成聚合物，NADH 脫氫酶及其鐵硫蛋白的氧化還原狀況能反映葉綠體之代謝狀況，而不同氧化還原狀況之鐵硫蛋白能影響 DNA 複製蛋白與 OriA 之親和性，進而調控 DNA 複製之起始。