

Zundel, Michael

Initiation of Chloroplast DNA Replication

Faculty Mentor: Brent Nielson, Microbiology and Molecular Biology

Background Information

Deoxyribonucleic acid (DNA) is the macromolecule found in cells that carries all of our genetic information. Eukaryotic cells contain a nucleus, where the DNA is housed. Plant cells also contain chloroplasts. Chloroplasts convert light energy into chemical energy, typically in the form of sugar. Chloroplasts contain DNA, which is thought to have evolved from an early symbiotic relationship between a eukaryotic cell and a prokaryotic cell, or bacterium. The eukaryotic cell engulfed the prokaryotic cell and used it for the conversion of light energy. Chloroplast DNA replicates independent of the cell cycle and the nuclear DNA.

In young seedlings, the number of chloroplast DNA (ctDNA) molecules may exceed 10,000 per cell, with up to 100 copies of the DNA in each of 100 chloroplasts per cell (Heinhorst and Cannon 1993, Heinhorst et al 1985, Tewari 1988). This high copy number provides an excellent opportunity for genetic engineering and recombinant DNA technology. These young plant cells could one day be used to produce human proteins, just as bacteria is used today to produce insulin.

Chloroplast DNA is organized as a circular molecule. Two replication origins have been mapped in pea and tobacco ctDNA, but the protein that actually initiates replication has not yet been identified. It is hypothesized that since the chloroplast has prokaryotic ancestry, the ctDNA will initiate replication in a similar fashion. The model for bacterial DNA replication initiation involves the DnaA protein (Schapter and Messer, 1995). This protein functions by binding to the DNA and positioning the DNA so the replication machinery can attach and begin to replicate.

In the lab of Dr. Brent Nielsen, two origins of replication (actually four, two of each in the large inverted repeats) have been mapped in the tobacco ctDNA genome, termed oriA and oriB (Kunnimalaiyaan and Nielsen, 1997). Replication originates at the oriA and oriB sites, forming two D-loops. These D-loops migrate towards each other until they meet, and replication is completed by a theta mechanism. Once two daughter strands are complete, replication may continue via a rolling circle mechanism (Kolodner and Tewari, 1975).

Materials and Methods

Chloroplasts were isolated from pea plants and then lysed, or broken open. Once lysis has occurred, the chloroplast lysate was subjected to ion-exchange chromatography. This two step purification process, first using a diethylaminoethyl (DEAE) cellulose resin, followed by a heparin sepharose resin. Heparin is a polyanion that mimics DNA. This second purification step will provide only proteins that bind to DNA. These chloroplast protein extracts were used for gel mobility shift assays and DNase footprinting assays.

Polymerase chain reaction (PCR) was performed to amplify the oriB region. Several different primer sets were designed to amplify different size fragments of oriB. All of these different sized fragments of DNA were used in gel mobility shift assays, along with the protein

extracts obtained from column chromatography. The gel mobility shift assay is possible since DNA migrates much slower through a polyacrylamide gel when it is bound to a protein than when it is not bound to a protein. The DNA fragments amplified from oriB were tested for binding affinity.

Once a DNA fragment is identified that has binding affinity for proteins extracted from the chloroplasts, the DNA fragment was used in an affinity pull-down assay. Affinity pull-down assay uses a DNA fragment and couples it to a magnetic bead. This bead is then used in further purification of the protein extract. Instead of getting all of the proteins that bind to DNA, only the proteins that bound to the specific fragment of oriB DNA were isolated. This purified protein product is now being analyzed by MALD-TOF Mass Spectroscopy in the Department of Biochemistry here on campus at BYU.

The same DNA fragment used for affinity chromatography was used for DNase footprinting. This assay uses DNase, a nuclease enzyme that randomly cleaves DNA, and protein extracts. The protein, when bound to the DNA, will protect it from nuclease activity. These fragments produced when digested by DNase are then run on a denaturing acrylamide gel. This creates a “ladder-like” pattern down the gel when no protein is bound to protect the DNA. The exact binding site of the protein was not able to be identified by the “footprint”, or area of the “ladder” that is missing and must be worked with further to obtain usable data.

Future Directions, Acknowledgements and Conclusion

The data from the mass spec analysis will be used to help identify the gene product(s) involved in the initiation of replication in chloroplast DNA. Once the footprinting assay is optimized, the actual binding site of the replication origin binding protein will also be elucidated.

This data was presented at the Experimental Biology show in San Diego, CA in April 2003 in the form of a poster. This poster is on display currently on the NW corner of the 8th floor of the Widtsoe Building. Without the ORCA funding, this work would not have progressed as far as it did.

Understanding the mechanism of ctDNA replication initiation will move us one step closer to our use of ctDNA in recombinant DNA technology. This will open the door for gene cloning in plant tissue, recombinant protein expression in plants, and many other possibilities that will become more lucid with time.

Works Cited

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