Planta (2001) 212: 851–857

Planta

Insertion of polydeoxyadenosine-rich sequences into an intergenic region increases transcription in *Chlamydomonas reinhardtii* chloroplasts

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Received: 8 June 2000 / Accepted: 8 August 2000

Abstract. In this study, chloroplast transformation in *Chlamydomonas reinhardtii* was used to insert a tract of polydeoxyadenosine, which is known to influence DNA structure and transcription in other systems, between the 3′ end of the *atpB* gene, encoding the β-subunit of the chloroplast ATP synthase, and a downstream chimeric gene, *aadA*, encoding antibiotic resistance. Run-on transcription and RNA analyses revealed that in cells containing $(dA)_{40}$ and $(dAAAGGG)_8$, *aadA* was transcribed at a higher rate, and its RNA accumulated to a relatively high level. It is concluded that poly(dA/dT) can function in the chloroplast as a transcription enhancer element. Therefore, the insertion of poly(dA/dT) sequence into the intergenic region of a multicistronic transcription unit may modulate gene expression at the transcriptional level.

Key words: Transcription (chloroplastic) – Chloroplast – Polydeoxyadenosine – Chloroplast transformation – *Chlamydomonas* (chloroplast transformation)

Introduction

The chloroplast genome of the green alga *Chlamydomonas reinhardtii* is similar to those of most vascular plants, which are circular 120- to 200-kb DNA molecules having two unique regions separated by large, inverted repeats (Sugiura 1992). The DNA molecule encodes about 120 proteins that are involved in the transcription, translation, photosynthesis and chlororespiratory processes (Sugiura 1992). Chloroplast genes are often found in clusters or polycistronic operons that are first co-transcribed and then undergo numerous processing events, which result in a complex mRNA accumulation pattern. The best-studied example in vascular plants is the *psbB* gene cluster, which encodes three photosystem II proteins (*psbB*, *psbT* and *psbH*) and two proteins of the cytochrome b_6/f complex (*petB* and *petD*) (Barkan 1988; Kohchi et al. 1988; Westhoff and Herrmann 1988). In maize and spinach, approximately 20 different RNA molecules are generated by processing the primary transcript initiated upstream of *psbB*, which includes two introns. In general, both transcriptional and post-transcriptional mechanisms play important roles in chloroplast gene regulation (Gruissem and Schuster 1993; Rochaix 1996; Sugita and Sugiura 1996; Drager and Stern 1998; Schuster et al. 1999).

The co-transcription of chloroplast genes is facilitated by the general absence of efficient transcription termination, as illustrated by a variety of in vitro and in vivo assays (Stern and Gruissem 1987; Stern and Kindle 1993; Rott et al. 1996). This, in turn, implies that not only can gene clusters be co-transcribed, but also that transcription may proceed through one gene cluster and into another. Such transcripts are indeed seen, for example, in chloroplast transformants of tobacco (Staub and Maliga 1994) or *Chlamydomonas* (Sakamoto et al. 1994), where selectable marker- or reporter-gene constructs have been introduced into intergenic regions.

A computer search of the complete sequence of the chloroplast genome of the green alga *Chlorella vulgaris* revealed 15–20 sequences having at least 12 adenosines or thymidines within 100 bp of promoters (Wakasugi et al. 1997). Nevertheless, it is unknown whether or not these sequences have any specific function. Poly(dA:dT) was reported to be a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure, and to influence gene expression when inserted into intergenic regions of yeast nuclear genes (Iyer and Struhl 1995) as well as the genome of *Chlamydia* (Schaumburg and Tan 2000). In a recent study analyzing the chloroplast *rbcL* (Rubisco large subunit) promoter in chloroplast transformants, the deletion of a poly(dA)-rich sequence

Abbreviations: ECS = endonuclease cleavage site; nt = nucleotides; PCR = polymerase chain reaction; UTR = untranslated region

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resulted in a slight (but not significant) reduction in transcription activity (Shiina et al. 1998). Does the poly(dA) tract inserted into the intergenic region of a chloroplast gene affect transcriptional activity? Our strategy was to insert a poly(dA) tract or control sequences between two genes, *atpB* and *aadA*. We found that transcription was increased for the downstream *aadA* gene, leading to the accumulation of an elevated amount of this transcript. These results demonstrate that the poly(dA) tract can function in the chloroplast as a transcription enhancer. The results also suggest that dA-rich intergenic sequences commonly found in the chloroplast genome may have heretofore unanticipated functions.

Materials and methods

Oligonucleotides

The oligonucleotides used for plasmid constructions were as follows:

(1) AG-ECS, 5′-GCGATC(AAAGGG)$_8$AATTTTTAGCATGTAAACATTAGAA
(2) A-ECS, 5′-GCGATC(A)$_{50}$TTTTTAGCATGTAAACATTAGAA
(3) N-ECS,5′-GCGATCCTAAAGGTATGGGTCACAACACTTACGGTGAACAGCTGCCCGGGATGCATTTTTAGCATGTAAACATTAGAA
(4) α-ECS, 5′-GCGATCGCTGGTATTTCTAATGTTTACATGCT
(5) atpA2, 5′-GCTAAAAGAGAAGAACAATGGG

where ECS is the endonuclease cleavage site.

Plasmids

The construction of plasmid pBSATΔ21, which contains the entire *atpB* gene and its 3′ untranslated region (UTR), has been described (Stern et al. 1991). The *aadA* cassette (atpX in Goldschmidt-Clermont 1991) was amplified by polymerase chain reaction (PCR) and inserted into the *Bgl*II and *Bam*HI restriction sites of pBSATΔ21. The resulting plasmid, with *aadA* downstream of *atpB*, was named pAC.

The plasmid used for the construction of the A strain was made using the oligonucleotides A-ECS and α-ECS. These two oligonucleotides were annealed, filled in with the Klenow fragment of DNA polymerase I, digested with *Sau*IIIA, and inserted into the *Bgl*II site of pAC, yielding pA. A DNA sequence analysis of pA revealed that only 40 adenosines were present (instead of 50 present in the oligonucleotide A-ECS). The pAG and pN plasmids were constructed similarly, using the AG-ECS and N-ECS oligonucleotides, respectively, instead of A-ECS. A DNA sequence analysis of these plasmids showed that, in these cases, the exact sequences of the oligonucleotides were cloned.

Chloroplast transformation

Chlamydomonas reinhardtii strain CC373, which lacks a functional *atpB* gene, was transformed using the Dupont particle delivery system with 2 μg of either pA, pAG or pN DNA as previously described (Stern et al. 1991). Phototrophic transformants were subcloned to generate homoplasmic strains.

Isolation of nucleic acids, filter hybridizations and PCR

DNA and RNA were isolated from cell cultures grown in 50 ml HSA medium to mid-log phase as previously described (Rott et al. 1998a). For RNA filter hybridizations, 10–20 μg total RNA was fractionated in 1.2% agarose 2.2 M formaldehyde gels, transferred to Amersham Hybond N Plus nylon membranes, and cross-linked by UV irradiation. Pre-hybridization and hybridization were conducted in 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M trisodium citrate), 50% formamide, 10× Denhardt's solution (100× Denhardt's = 2% BSA, 20% Ficoll 400, 2% polyvinylpyrrolidone), 0.1% SDS and 100 μg/ml carrier salmon sperm DNA at 42 °C. Hybridization probes were generated from gel-purified linear double-stranded DNA fragments by random priming in the presence of α-[^{32}P]dCTP. The gene-specific probes used were: *Eco*RI/*Bgl*II, 2,000 bp of the pΔ26 plasmid for *atpB* (Stern et al. 1991); the 800-bp *Eco*RI/*Pst*I fragment of the chloroplast genomic fragment p86-Eco22 for *atpA* (Drapier et al. 1998); the 800-bp *Sac*I/*Xba*I fragment of the *aadA* gene (Goldschmidt-Clermont 1991); and the end-labeled α-ECS oligonucleotide for *petD*.

Analysis of DNA structure

The intergenic regions were PCR-amplified from the plasmids pA, pAG and pN described above using the primers DBS4 (Stern et al. 1991) and atpA2. The PCR products were labeled with α-[^{32}P]ATP and T4 polynucleotide kinase. The ligated product of a 100-bp ladder labeled in the same way was used for size markers. The PCR products were resuspended in a buffer containing 50 mM Tris pH 7.6, 10 mM $MgCl_2$, and 5 mM dithiothreitol and electrophoresed in 8% polyacrylamide gels (acrylamide:bisacrylamide 29:1) at 25 °C or 4 °C (Koo et al. 1986; Koo and Crothers 1988). Gels were dried and autoradiographed.

Run-on transcription assays

Permeabilized cells for run-on transcription were prepared using the freeze/thaw method, as previously described, and transcripts were labeled with α-[^{32}P]UTP and purified (Rott et al. 1996). The purified radioactively labeled RNA was hybridized to a filter-bound 800-bp *Sac*I/*Xba*I fragment of the *aadA* gene (Goldschmidt-Clermont 1991), the *Eco*RI/*Bgl*II 2,000-bp fragment of the pΔ26 plasmid for *atpB* (Stern et al. 1991), and the *Eco*RI fragment of the *psbA* gene (Rott et al. 1998b). Membranes were hybridized and washed as previously described (Rott et al. 1998b), and quantified using a Fuji imaging analyzer.

Results

Construction of Chlamydomonas strains containing intergenic insertions

To test the effects of A-rich sequences on chloroplast gene expression, chloroplast transformants were constructed according to the scheme illustrated in Fig. 1. The insertions were made downstream of the endogenous *atpB* gene, encoding the β-subunit of the chloroplast ATP synthase, and upstream of the chimeric transgene containing the *aadA* coding region, an *Escherichia coli* gene that confers resistance to the antibiotics spectinomycin and streptomycin (Goldschmidt-Clermont 1991). Since these two genes are in tandem, the insertions lie immediately downstream of

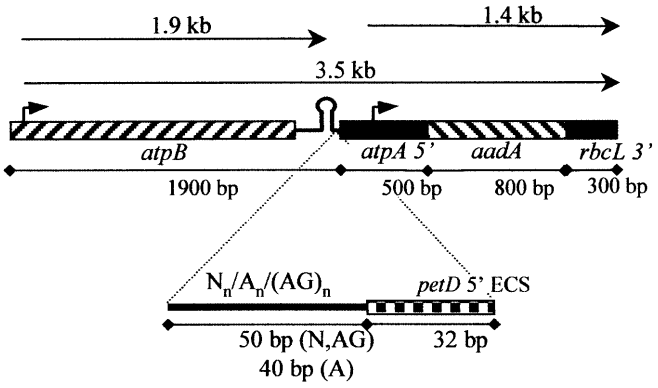

Fig. 1. The *Chlamydomonas* chloroplast constructs used in this work. A schematic representation of the *atpB* gene, A, AG, N, and ECS (*petD* 5′ UTR endonuclease cleavage site) insertions, and *aadA* cassette used to create the transformants. The *atpB* and *aadA* coding regions are depicted by *hatched boxes*. The inverted repeats located in the 3′ UTR of *atpB* are symbolized by a stem-loop structure. The *atpA* promoter/5′ UTR and *rbcL* 3′ UTR located in the *aadA* cassette are depicted by *filled boxes*. Promoters are symbolized by *bent arrows*, and the *arrows at the top* indicate the predicted RNA molecules and approximate sizes

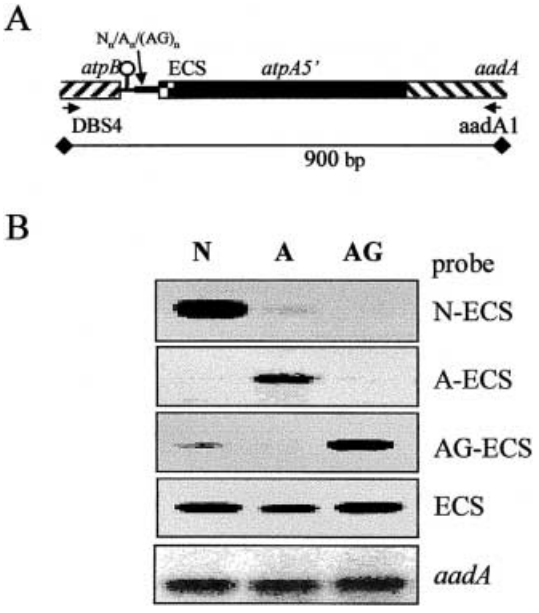

Fig. 2A,B. Analysis of the *Chlamydomonas* transformants by PCR. **A** Map of the region between the *atpB* gene and the *aadA* cassette in the transformants. Symbols are as in Fig. 1. Primers used for PCR amplification are marked by *arrows*. **B** DNA was isolated from the indicated strains and amplified by PCR using the primers DBS4 and addA1. The PCR products were resolved in agarose gels, transferred to nylon membranes, and hybridized with probes of 82 nt covering the N, A and AG sequences, in addition to the ECS, the ECS (32 nt) or the *aadA* coding sequence

the 3′ end of the mature *atpB* mRNA defined by a stem-loop structure (Stern and Kindle 1993), and 180 nucleotides (nt) upstream of the DNA sequence containing the *atpA* promoter, which drives *aadA* transcription. The insertion cassette contained A_{40}, $(AAAGGG)_8$ or 50 randomized nucleotides (N_{50}), followed by a 32-bp sequence derived from the 5′ UTR of the *petD* gene (see the sequence of the N-ECS oligonucleotide in *Materials and methods*). The random sequence was used as a "spacer" control.

The recipient strain CC373 was used to introduce these chimeric cassettes into *Chlamydomonas*. CC373 harbors a deletion of most of the *atpB* coding region and is therefore non-photosynthetic. CC373 cells were restored to photosynthetic competence following biolistic transformation, and screened for homoplasmic insertion of each cassette (see below) with strains containing A_{40}, $(AAAGGG)_8$, or 50 randomized nucleotides between *atpB* and *aadA* referred to as A, AG and N, respectively. We expected that even if 3′ UTR-mediated stabilization of *atpB* mRNA was affected, sufficient protein would be produced to enable photosynthetic growth, since even unstable *atpB* mRNA lacking the entire 3′ UTR supports photosynthesis (Stern et al. 1991). On the other hand, we were uncertain what expression level to expect from the *aadA* cassette and did not want to select it for its function.

As expected, transformants were obtained in all cases, and their photosynthetic growth rates on solid minimal medium were indistinguishable from those of wild-type cells. To verify that the strains contained the correct insertions, DNA filter hybridizations, DNA sequencing of the intergenic region, and PCR amplification were carried out. Figure 2 shows that when total DNA was isolated from the transformants and amplified by PCR, 900-bp products were obtained that hybridized with probes covering the inserted sequences in the appropriate pattern. Cross-hybridization between the N, A and AG probes was minimal, with weak signals due to the 32-bp ECS signal present in all the probes and genomes. However, probes specific for the ECS or the *aadA* gene hybridized equivalently with the PCR products from all of the strains. These results are consistent with chloroplast transformants having the expected DNA insertions.

DNA structures in the intergenic regions

A stretch of dA:dT was previously reported as being an element that alters the classic double-helical DNA structure, leading to changes in promoter activity (Iyer and Struhl 1995; Koo et al. 1986). In order to analyze the structures of our chimeric intergenic regions, these sequences were PCR-amplified and electrophoresed in polyacrylamide gels (Koo et al. 1986; Koo and Crothers 1988). When analyzed in a denaturing gel, the three PCR products migrated as discrete and well-defined bands according to their molecular weights (Fig. 3A). However, when analyzed under non-denaturing conditions, the DNA containing A_{40} generated a heterodisperse pattern of a higher molecular weight compared to the random sequence N (Fig. 3B). The DNA containing $(AAAGGG)_8$ also showed aberrant migration, albeit to a lesser extent than the A_{40} sequence. The migration anomalies result from structural alterations in the DNA molecules, and since the only differences between the DNA molecules were the 40- or 50-bp stretches with A-rich

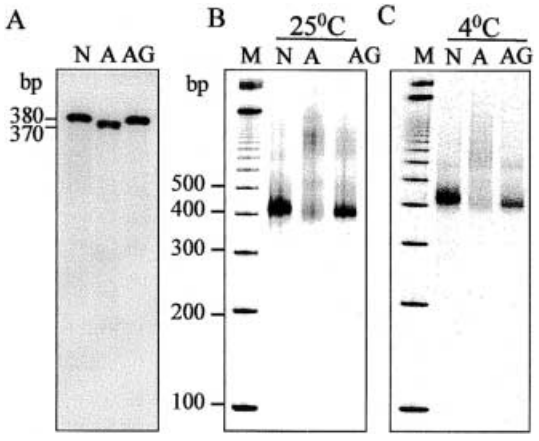

Fig. 3A–C. Conformational variation of DNA in the intergenic regions. The intergenic regions of the plasmids used for transformation, pA, pAG and pN, were PCR-amplified using primers DBS4 and atpA2, followed by ^{32}P end-labeling. The DNA fragments were then fractionated in a denaturing polyacrylamide-urea gel (**A**), or non-denaturing acrylamide gels that were run either at 25 °C (**B**) or 4 °C (**C**). A ligated ^{32}P-labeled 100-bp ladder was used as size markers (*lanes M*). The lengths of the PCR products were 380 bp for N and AG, and 370 bp for A

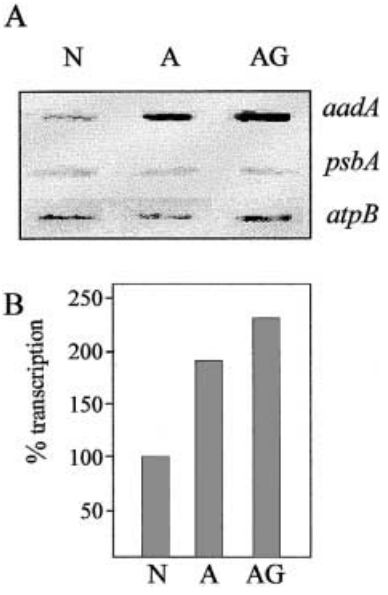

Fig. 4A, B. Analysis of the transcription rate of *aadA* RNA in the *Chlamydomonas* chloroplast transformants. **A** ^{32}P-labeled RNAs isolated from freeze-thaw-permeabilized strains as indicated were hybridized with filters containing DNA fragments corresponding to *aadA, psbA* and *atpB*. **B** The relative transcription rates of the *aadA* gene were quantified using a Fuji imaging analyzer and normalized to the corresponding values of the *psbA* mRNA. The transcription value of the *aadA* gene in the N strain was set to 100%

or random sequences, these sequences must be responsible. However, there was a possibility that the migration anomaly was induced by the opening of the relatively weak A-T bonds in the A_{40} stretch during gel electrophoresis, and that it would be unrelated to the native structure of the DNA in the chloroplast. To test this possibility, NaCl and $MgCl_2$ were added to concentrations of 100 mM and 10 mM, respectively. However, the same result, as illustrated in Fig. 3B, was obtained (data not shown). In addition, reducing the temperature of gel electrophoresis to 4 °C had no effect, as shown in Fig. 3C. Therefore, these results demonstrate that A_{40}, and to a lesser extent $(AAAGGG)_8$, induce conformational changes in the intergenic regions analyzed in this work.

Increased transcription activity in strains A and AG leads to increased accumulation of aadA *mRNA*

In order to determine whether the different insertions modulated the transcription activity of the *aadA* gene, run-on assays using freeze-thaw permeabilized *Chlamydomonas* cells were performed (Rott et al. 1996). Transcripts were labeled for 15 min with [^{32}P]UTP and hybridized with filter-bound DNA fragments derived from the *aadA* and *atpB* coding regions and from another distantly located chloroplast gene, *psbA*, as a control. Figure 4A shows the results of one such experiment; the quantified hybridization signals are illustrated in Fig. 4B. When the *aadA* transcription rate was normalized to that of *psbA*, and the rate in the control (N) strain was set to 100%, the transcription rate in the A and AG strains was found to be roughly doubled. No significant differences in the transcription activity of the upstream *atpB* gene were observed (Fig. 4A). The results for *aadA* are in agreement with those obtained in yeast, where the insertion of poly(dA:dT) stimulated transcription by RNA polymerase II by 3- to 6-fold (Iyer and Struhl 1995). In addition, it was reported that the transcription of the T7 RNA polymerase, when inserted into yeast cells, is driven by the poly(dA:dT) element in the *DED1* promoter (Chen et al. 1987). The results obtained in this work suggest that the chloroplast RNA polymerase involved in the transcription of *aadA* may react in a similar way.

To determine whether the differences in transcription rates resulted in a differential accumulation of *aadA* transcripts, total RNA was analyzed by filter hybridization with gene-specific probes for *aadA*, and the ECS sequence which detects mainly the *petD* transcript, and was used as loading control. Since the ECS sequence is derived from the *petD* 5′ UTR, this probe would identify the normal *petD* transcript. As shown in Fig. 5, the ECS probe revealed similar amounts of the 0.9-kb *petD* transcript. However, in agreement with the increased transcription activity shown in Fig. 4, the accumulation of the major *aadA* transcript of about 1.4 kb was markedly higher in strains A and AG, as much as 10-fold over the amount in the control strain N. These results showed that the increased transcription rate of the *aadA* gene in the A and AG strains correlated with, and probably accounted for, the accumulation of an elevated steady-state amount of *aadA* transcripts. However, the differences between the transcription rates (×2) as compared to the transcript accumulation (×10), suggested that either they are not strictly coupled, or that the insertion of poly(dA;dT) also affected RNA stability, in addition to transcription rate.

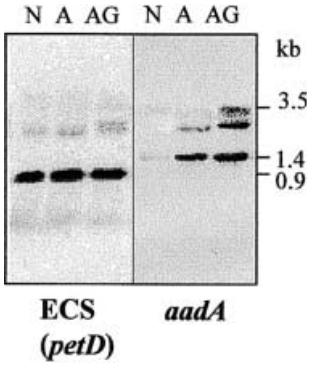

Fig. 5. Accumulation of the *aadA* and *atpB* mRNAs in the *Chlamydomonas* chloroplast transformants. Total RNA (20 μg) from the indicated transformants was fractionated in agarose gels and hybridized sequentially with ^{32}P-labeled *petD*-ECS and *aadA* probes as described in *Materials and methods*. Sizes of the hybridizing species are indicated on the *right*

Discussion

In this study, we have shown that the insertion of a short intergenic sequence consisting of poly(dA) or poly(dA + dG) can affect chloroplast RNA synthesis. The influence of adjacent genes and intergenic regions on gene expression at transcriptional levels is an important issue for relatively compressed genomes such as those of bacteria, mitochondria, chloroplasts and yeast (Proudfoot 1986; Henderson et al. 1989; Greger and Proudfoot 1998; Schaumburg and Tan 2000). For example, the importance of the poly(A) signal in controlling transcription termination and initiation between the tandem *GAL10* and *GAL7* genes of *Saccharomyces cerevisiae* has recently been described (Greger and Proudfoot 1998). In this case, it was shown that the deletion of the *GAL10* poly(A) signal resulted in complete inactivation of the *GAL7* promoter, causing a dramatic increase in bicistronic *GAL10-7* mRNA. Several characteristics of the chloroplast genomic environment make it a good system for analyzing the roles of intergenic sequences in gene expression. The first is that the chloroplast genes are closely spaced and their expression signals may overlap (Sugiura 1992). The second is that many chloroplast genes are organized in multicistronic units, and multicistronic mRNAs are easily detected (Barkan 1988; Westhoff and Herrmann 1988; Sugiura 1992; Drapier et al. 1998). Third, it was shown that stem-loop structures located at the 3′ ends of most chloroplast transcripts are processing signals rather than transcription terminators (Stern and Gruissem 1987; Stern et al. 1989; Stern and Kindle 1993; Rott et al. 1996, 1998b). This leads to a situation whereby intercistronic regions must contain accurate RNA processing signals and might have other unknown functions. Fourth, chloroplast transformation makes it possible to manipulate these intergenic regions. Our strategy in this study was to add a potential transcription enhancer sequences, or related controls, upstream of the *atpA* promoter, which is driving the expression of an antibiotic resistance gene. Effects on transcription that led to modulation transcript accumulation were detected for the *aadA* gene. The relative transcription activity of the *aadA* gene was doubled in strains A and AG (Fig. 4), resulting in the accumulation of considerably more monocistronic *aadA* transcripts (Fig. 5). It should be noted however, that only the atpA promoter was analyzed in this work. Since different type of promoters and several DNA-dependent RNA-polymerases are involved in chloroplast transcription, analyzing other promoters at different locations along the chloroplast chromosome is required in order to generalize the phenomenon observed in this work.

We do not yet know how A_{40} and $(AAAGGG)_8$ cause increased transcription. One possibility is that changes in DNA conformation (Fig. 3) led to increased promoter activity, as described for yeast (Iyer and Struhl 1995). This phenomenon has been described in vivo in *Chlamydomonas* chloroplasts with the addition of the DNA gyrase inhibitor novobiocin (Thompson and Mosig 1985, 1987). The role of DNA conformation in chloroplast transcription initiation has also been seen using in vitro transcription systems (Stirdivant et al. 1985; Lam and Chua 1987). Furthermore, a more recent study by Salvador et al. (1998) found a positive correlation between in vivo DNA supercoiling and transcription of *atpB* in *Chlamydomonas*, supporting the idea that DNA conformation influences chloroplast transcription. The *atpA* promoter used in our *aadA* cassette was not examined at the transcriptional level by Salvador et al. (1998); however, endogenous control of its local superhelicity was observed. If DNA-conformation changes indeed alter transcription, this might be modulated by certain DNA-binding proteins with an affinity for the A or A + G motifs. An example of such a protein is the barley chloroplast transcription factor AGF, which is involved in the blue-light-stimulated transcription of *psbD* and binds to a sequence with multiple AAG motifs (Kim and Mullet 1995), not unlike the AG sequence introduced in our study. However, it has been reported that in *E. coli* transcriptional enhancement mediated by upstream A-tracts is be mediated by direct interaction with the RNA polymerase (Aiyar et al. 1998). In addition, in a recent study in which a poly(dA)-rich sequence was eliminated from the promoter of the *rbcL* in tobacco chlorotransgenes, the promoter activity was not significantly reduced (Shiina et al. 1998). It is possible that the length and location of the poly(dA) tract are crucial factors that determine whether or not it will enhance transcription.

As described above, a computer search of the complete sequence of the chloroplast genome of the green alga *Chlorella vulgaris* revealed 15–20 sequences of at least 12 adenosines or thymidines within 100 bp of promoters (Wakasugi et al. 1997). It will be interesting to explore if these sequences modulate transcription for the *Chlamydomonas* chloroplast, as shown in this study. In addition, it will open the way to use this as a *cis* element for the genetic engineering of chlorotransgenes aimed at producing highly valuable protein in the chloroplast.

Overall, our data suggest that the genome context is important for chloroplast gene expression, in addition to the well-known contributions of *cis* elements such as

promoters and translation elements. This phenomenon is better characterized in plant nuclei, where position effects can dramatically alter the expression of transgenes. However, the influence of non-promoter elements on the transcription of chloroplast genes has been documented in *Chlamydomonas*, where sequences within the *rbcL* coding region were found to enhance transcription (Klein et al. 1994). Future studies may reveal other regulatory aspects of the poorly studied, A + T-rich intergenic sequences found in *Chlamydomonas* and other chloroplast genomes.

We thank Robert Drager for contributing to the inception of this project and David Stern (Boyce Thompston Institute, Cornell University, New York) for his numerous helpful comments and advice. We thank Tali Haran and members of her laboratory for their assistance in planning the experiments to analyze DNA structure (Fig. 3) and Ilana Cohen for preparing the DNA markers. This work was supported by the United States – Israel Binational Agricultural Research and Development Fund and United States – Israel Binational Science Foundation grants.

References

Aiyar SE, Gourse RL, Ross W (1998) Upstream A-tracts increase bacterial promoter activity through interactions with the RNA polymerase alpha subunit. Proc Natl Acad Sci USA 95: 14652–14657

Barkan A (1988) Proteins encoded by a complex chloroplast transcription unit are each translated from both monocistronic and polycistronic RNAs. EMBO J 7: 2637–2644

Chen W, Tabor S, Struhl K (1987) Distinguishing between mechanisms of eukaryotic transcriptional activation with bacteriophage T7 RNA polymerase. Cell 50: 1047–1055

Drager RG, Stern DB (1998) Chloroplast RNA synthesis and processing. In: Rochaix J-D, Goldschmidt-Clermont M, Merchant S (eds) Molecular biology of *Chlamydomonas*: chloroplasts and mitochondria. Kluwer, Dordrecht, pp 165–140

Drapier D, Suzuki H, Levy H, Rimbault B, Kindle KL, Stern D, Wollman FA (1998) The chloroplast *atpA* gene cluster in *Chlamydomonas reinhardtii*. Functional analysis of a polycistronic transcription unit. Plant Physiol 117: 629–641

Goldschmidt-Clermont M (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. Nucleic Acids Res 19: 4083–4090

Greger IH, Proudfoot NJ (1998) Poly(A) signals control both transcriptional termination and initiation between the tandem *GAL10* and *GAL7* genes of *Saccharomyces cerevisiae*. EMBO J 17: 4771–4779

Gruissem W, Schuster G (1993) Control of mRNA degradation in organelles. In: Brawerman G, Belasco J (eds) Control of messenger RNA stability. Academic Press, Orlando, pp 329–365

Henderson SL, Ryan K, Sollner-Webb B (1989) The promoter-proximal rDNA terminator augments initiation by preventing disruption of the stable complex caused by polymerase read-in. Genes Dev 3: 212–223

Iyer V, Struhl K (1995) Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. EMBO J 14: 2570–2579

Kim M, Mullet JE (1995) Identification of a sequence-specific DNA binding factor required for transcription of the barley chloroplast blue light-responsive *psbD-psbC* promoter. Plant Cell 7: 1445–1457

Klein U, Salvador ML, Bogorad L (1994) Activity of the *Chlamydomonas* chloroplast *rbcL* gene promoter is enhanced by a remote sequence element. Proc Natl Acad Sci USA 91: 10819–10823

Kohchi T, Yoshida T, Komano T, Ohyama K (1988) Divergent messenger RNA transcription in the chloroplast *psbB* operon. EMBO J 7: 885–892

Koo H, Wu H, Crothers DM (1986) DNA bending at adenine-thymine tracts. Nature 320: 501–506

Koo H-S, Crothers D (1988) Calibration of DNA curvature and a unified description of sequence-directed bending. Proc Natl Acad Sci USA 85: 1763–1767

Lam E, Chua N-H (1987) Chloroplast DNA gyrase and *in vitro* regulation of transcription by template topology and novobiocin. Plant Mol Biol 8: 415–424

Proudfoot NJ (1986) Transcriptional interference and termination between duplicated alpha-globin gene constructs suggests a novel mechanism for gene regulation. Nature 322: 562–565

Rochaix J-D (1996) Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. Plant Mol Biol 32: 327–341

Rott R, Drager RG, Stern DB, Schuster G (1996) The 3′ untranslated regions of chloroplast genes in *Chlamydomonas reinhardtii* do not serve as efficient transcriptional terminators. Mol Gen Genet 252: 676–683

Rott R, Levy H, Drager RG, Stern DB, Schuster G (1998a) 3′-Processed mRNA is preferentially translated in *Chlamydomonas reinhardtii* chloroplasts. Mol Cell Biol 18: 4605–4611

Rott R, Liveanu V, Drager RG, Stern DB, Schuster G (1998b) The sequence and structure of the 3′-untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability. Plant Mol Biol 36: 307–314

Sakamoto W, Sturm NR, Kindle KL, Stern DB (1994) *petD* mRNA maturation in *Chlamydomonas reinhardtii* chloroplasts: The role of 5′ endonucleolytic processing. Mol Cell Biol 14: 6180–6186

Salvador ML, Klein U, Bogorad L (1998) Endogenous fluctuations of DNA topology in the chloroplast of *Chlamydomonas reinhardtii*. Mol Cell Biol 18: 7235–7242

Schaumburg CS, Tan M (2000) A positive *cis*-acting DNA element is required for high level transcription in *Chlamydia*. J Bacteriol 172: 5167–5171

Schuster G, Lisitsky I, Klaff P (1999) Update on chloroplast molecular biology: polyadenylation and degradation of mRNA in the chloroplast. Plant Physiol 120: 937–944

Shiina T, Allison L, Maliga P (1998) *rbcL* transcript levels in tobacco plastids are independent of light: reduced dark transcription rates is compensated by increased mRNA stability. Plant Cell 10: 1713–1722

Staub JM, Maliga P (1994) Translation of *psbA* mRNA is regulated by light via the 5′-untranslated region in tobacco plastids. Plant J 6: 547–553

Stern DB, Gruissem W (1987) Control of plastid gene expression: 3′ inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. Cell 51: 1145–1157

Stern DB, Kindle KL (1993) 3′ end maturation of the *Chlamydomonas reinhardtii* chloroplast *atpB* mRNA is a two-step process. Mol Cell Biol 13: 2277–2285

Stern DB, Jones H, Gruissem W (1989) Function of plastid mRNA 3′ inverted repeats: RNA stabilization and gene-specific protein binding. J Biol Chem 264: 18742–18750

Stern DB, Radwanski ER, Kindle KL (1991) A 3′ stem/loop structure of the *Chlamydomonas* chloroplast *atpB* gene regulates mRNA accumulation in vivo. Plant Cell 3: 285–297

Stirdivant SM, Crossland LD, Bogorad L (1985) DNA supercoiling affects in vitro transcription of two maize chloroplast genes differently. Proc Natl Acad Sci USA 82: 4886–4890

Sugita M, Sugiura M (1996) Regulation of gene expression in chloroplasts of higher plants. Plant Mol Biol 32: 315–326

Sugiura M (1992) The chloroplast genome. Plant Mol Biol 19: 149–168

Thompson RJ, Mosig G (1985) An ATP-dependent supercoiling topoisomerase of *Chlamydomonas reinhardtii* affects accumulation of specific chloroplast transcripts. Nucleic Acids Res 13: 873–891

Thompson RJ, Mosig G (1987) Stimulation of a *Chlamydomonas* chloroplast promoter by novobiocin in situ and in *E. coli* implies regulation by torsional stress in the chloroplast DNA. Cell 48: 281–287

Wakasugi T, Nagai T, et al (1997) Complete nucleotide sequence of the chloroplast genome from the green alga *Chlorella vulgaris*: the existence of genes possibly involved in chloroplast division. Proc Natl Acad Sci USA 94: 5967–5972

Westhoff P, Herrmann RG (1988) Complex RNA maturation in chloroplasts: the *psbB* operon from spinach. Eur J Biochem 171: 551–564